DIAGNOSTIC
LABORATORY
HEMATOLOGY

IAGNOSTIC
ABORATORY
IEMATOLOGY

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SECOND EDITION
Revised and Enlarged



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Preface to the Second Edition

In the preparation of the second edition, it has been difficult for the author to adhere completely to the purpose of the first edition and at the same time keep pace with the increasing importance of the laboratory in the diagnosis of disease. Included in the second edition are methods, such as the determination of hemoglobin, heme pigments in serum, osmotic fragility and serum bilirubin which require the use of a photometer. A method for counting platelets with a phase contrast microscope and procedures for the identification of abnormal hemoglobins by paper electrophoresis have been added. Admittedly, these methods require the use of more than simple, inexpensive apparatus. They have been added because they are considerably more accurate than are those which require less expensive apparatus and because many small diagnostic laboratories now have the necessary equipment. The methods which require less expensive apparatus have been retained so that the student of medicine, medical technologist, or physician, for whom this manual is written, may choose whichever method best suits his or her conditions.

The sections concerning tests for hemolytic and hemorrhagic disorders have been expanded considerably. In general, these tests are easy to perform and a minimum of equipment is required. However, the intelligent application of these procedures and the interpretation of the results require some understanding of hemolytic and hemorrhagic disorders. For this reason, a small amount of explanatory material has been added as an introduction to these sections and, for the same reason, an explanatory introduction has been added to the sections on blood and urine pigments related to hemoglobin

Data concerning the accuracy of a number of methods, when performed by medical students with limited experience, have been included in this edition Such information should be of value and interest to both teachers and students

The technics are presented, as in the first edition, in a stepwise fashion and emphasis is placed on small technical details and on possible sources of error. To the author, it seems better to present methodology in this "cook book" fashion than to outline it sketchily, omitting small but important details. The latter type of descrip-

tion is to be found all too commonly in the American literature today.

The author is greatly indebted to his chief, Dr. M. M. Wintrobe, for his "hematologic upbringing" and for his constant advice and criticism. For the illustrative material, he owes thanks to his wife, Helene, and to Mr. Howard Tribe. To his medical technologist, Miss Doris Kurth, and to his secretary, Miss Bonnie Jo Nelson, sincere appreciation is due.

G. E. CARTWRIGHT, M.D.

Salt Lake City
September, 1957

Preface to the First Edition

The purpose of this manual is to present in concise form the essential details for the performance and critical evaluation of procedures used commonly in student laboratories and doctors' offices for the diagnosis of hematologic disorders.

For each determination a multiplicity of technics is available. As a general rule, the more numerous the technics available, the less satisfactory is the determination. The student, house staff officer, and practicing physician for whom this manual is written have neither the time nor the experience to evaluate all of the modifications suggested in the great mass of literature. Therefore, in each case, a single method for each determination has been selected on the basis of the author's experience. Simplicity, accuracy, reliability, time of performance, cost and availability of equipment are all factors which have been taken into consideration in making the selection. When a variety of technics of equal merit and demerit have been available to the author for selection, the simplest method to perform has always been selected. However, only those methods have been selected which can be performed in student laboratories and doctors' offices with a minimum of low cost equipment and a minimum of skill and experience. In many cases this has not resulted in the selection of the most specific, accurate, or best available method, but the most practical method under the circumstances For more specific and elaborate methods, *A Syllabus of Laboratory Examinations in Clinical Diagnosis*, edited by T H. Ham, Harvard University Press, Cambridge, Massachusetts, should be consulted

For corollary reading concerning the clinical aspects of hematology, *Clinical Hematology* by M. M. Wintrobe, Lea and Febiger, Philadelphia, Pa., is recommended.

An appendix concerning the preparation of reagents has been included because when former students have found themselves responsible for their own office laboratory, they frequently have found it necessary to inquire concerning these rather elementary and yet important matters.

Salt Lake City
January, 1954

G E CARTWRIGHT, M D.

Statistical Considerations

"The element of error cannot be eliminated from our observations and our reasonings. The only true scientific method is to study it."—J. F. Merz.

In clinical hematology, as in medicine, the clinician's primary interest is in significant deviations from the normal, that is, disease. Unfortunately, because of technical as well as biologic variation, it is not possible to deal in absolute or true values. The best that can be obtained is a series of approximations to the correct value. That is to say, the probable limits within which the true value lies are defined, rather than the actual value itself. Therefore, to determine if a given value is abnormal, it is imperative to know the limits of the "normal" variation and the possible error of the value which is to be compared to the "normal." For this it is necessary to understand a few simple statistical concepts.

Average or Mean. The average or mean ($\bar{X}$) is defined as the sum of all of the observations (SX) divided by the number of observations (N).

$$\bar{X} = \frac{SX}{N}$$

$\bar{X}$ = the mean
X = the various individual measurements
S = means take the sum of ________
N = the number of determinations.

Standard Deviation. The standard deviation (S.D.) is a measure of the variability about the mean. One standard deviation on each side of the mean (± 1 S.D.) is defined as that value which takes in approximately 68 per cent of the determinations. Two standard deviations on each side of the mean (± 2 S.D.) are defined as that value which takes in 95 per cent of the determinations. The standard deviation is expressed in the same units as the quantity measured.

$$\text{S.D.} = \sqrt{\frac{S(X - \bar{X})^2}{(N - 1)}}$$

Coefficient of Variation. The coefficient of variation (C.V.) is the standard deviation (S.D.) expressed as per cent of the mean. It is useful in comparing the standard deviations of quantities which are expressed in different units.

$$C.V. = \frac{S.D.}{\bar{X}} \times 100$$

An example of the above calculations is given in table 1.

TABLE 1—*Calculation of the Mean, Standard Deviation and Coefficient of Variation*

Determination N	Red Cell Count $10^6/mm^3$ X	$X - \bar{X}$	$(X - \bar{X})^2$
1	5.20	0.20	.04
2	4.80	0.20	.04
3	5.55	0.55	.30
4	5.10	0.10	.01
5	5.60	0.60	.36
6	4.10	0.60	.36
7	5.30	0.30	.09
8	4.45	0.55	.30
9	4.70	0.30	.09
10	4.90	0.10	.01
SX	50.00		1.60

$N = 10$

$$\bar{X} = \frac{SX}{N} = \frac{50}{10} = 5.00$$

$$S.D. = \sqrt{\frac{S(X-\bar{X})^2}{(N-1)}} = \sqrt{\frac{1.60}{9}} = \sqrt{0.178} = 0.42$$

$$C.V. = \frac{0.42}{5.00} \times 100 = \pm 8.4 \text{ per cent}$$

REFERENCE

An excellent reference on the errors of a number of routine hematologic methods is as follows: BIGGS, R. AND MACMILLAN, R. L., The errors of some haematological methods as they are used in a routine laboratory. J. Clin. Path. 1: 269, 1948.

The Equipment and Its Care

Hemocytometer. The hemocytometer consists of two chambers separated from one another by a transverse trench and bordered bilaterally by a longitudinal trench. The "improved Neubauer" ruling is used. Each chamber is 3 x 3 x 0.1 mm. and has a volume of 0.9 mm.³ The ruled area consists of 9 large squares, each 1 x 1 x 0.1 mm. with a volume of 0.1 mm.³ and bordered by a triple line. The center line of the three is the boundary line of the square. Each of the 4 corner squares is subdivided into 16 smaller squares measuring 0.25 x 0.25 x 0.1 mm. with a volume of 0.00625 mm.³ The center square of the 9 large squares is divided in 25 smaller squares each measuring 0.2 x 0.2 x 0.1 mm. with a volume of 0.0040 mm.³ (fig. 1).

The cover glass used over the chambers is made with precision. According to U. S. Bureau of Standards requirements the cover glass must be free of visible defects and must be optically plane on both sides within ± 0.002 mm. From this it is evident that the cover glass must be handled carefully like any other piece of calibrated microchemical equipment. Obviously, ordinary cover glasses cannot be substituted.

To clean the hemocytometer and cover glass, rinse immediately after use in cold or lukewarm water. Never use hot water. Never allow the diluted blood to dry on the chamber. If the diluted blood has been allowed to dry on the chamber, it may be washed off with soap and water (lukewarm), gently using the ball of the thumb to accelerate its action. Then thoroughly wash and rinse in running water. A soft cloth may be used to wipe off the water. The chamber and cover glass are allowed to dry in air. Before using, the chamber and cover glass must be absolutely clean and free from lint and water marks. Never touch the chamber or cover glass except at the edges after it has been cleaned. A scratch across the chamber or cover glass renders it useless.

Pipets. Under the liberal U. S. Bureau of Standards specifications, the allowable error of the red cell pipet is ± 5 per cent; of the hemoglobin pipet, ± 5 per cent; and of the white cell pipet, ± 3.5 per cent. Many manufacturers are now marketing all three types of pipets which they "guarantee" to be accurate within ± 1 per cent.

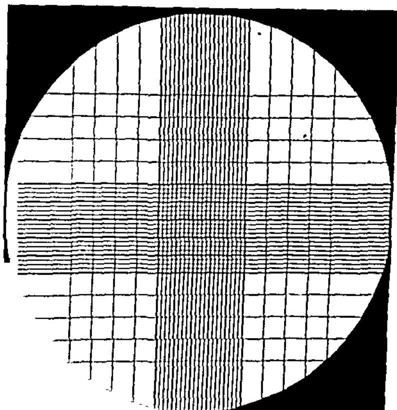


Fig 1.—The Improved Neubauer ruling of the counting chamber

The results of calibration by the National Bureau of Standards of 36 red cell pipets are shown in table 2. These pipets were purchased on the "open market" and all were "guaranteed" by

TABLE 2—*Results of Calibration of 36 Red Blood Cell Dilution Pipets*

Error* %	No of Pipets Brand A	Brand B	Brand C	Total
±1	4	4	0	8
±5	8	4	3	15
±10	0	4	1	8
±15	0	0	2	2
±25	0	0	3	3

*of a 1:200 dilution.

the manufacturer to be accurate within ± 1 per cent. From these results it is apparent that the manufacturer's "guarantee" cannot be relied upon and there is a possibility that a pipet may be purchased which has a gross dilution error. Therefore, it is recommended that pipets be purchased which have been certified by the National Bureau of Standards to be accurate within ± 5 per cent, or better still to purchase red cell pipets and then send them to the National Bureau of Standards* for calibration. The correction factor for both a 1:100 and a 1:200 dilution will be obtained and can be used.

The results of calibration by the National Bureau of Standards of 62 Sahli hemoglobin pipets are shown in table 3. These pipets were purchased on the "open market" and all were "guaranteed" by the manufacturer to be accurate within ± 1 per cent. Less expensive pipets may vary as much as ± 30 per cent of the true volume.

TABLE 3—*Results of Calibration of 62 Sahli Hemoglobin Dilution Pipets*

Brand	Error		
	± 1%	± 2%	± 3%
A	7	3	2
B	9	1	0
C	8	1	1
D	10	0	0
E	14	6	0
Total	48	11	3

The figures refer to number of pipets

Because a high degree of accuracy is not required of the leukocyte count for clinical purposes, it is usually adequate to purchase pipets which are stated to be accurate within ± 3 5 per cent.

The marks on the red and white cell pipets are arbitrary in order to accomplish a certain dilution of the sample when the pipet is filled The volume of the red cell pipet is made up of 1 part in the capillary portion and 100 parts in bulb When blood is drawn to the

*Capacity, Density and Fluid Meters Section, National Bureau of Standards, Washington, D C Although there is a modest charge for this service, for a small laboratory it is usually less expensive and more satisfactory to send them to the N B S than to do one's own calibration

0.5 mark and subsequently diluted to the 101 mark, all of the red cells are washed into the bulb, thus making a dilution of 0.5 parts in 100 parts of the bulb (1/200), the cell free contents in the capillary portion not participating in the dilution. In the white cell pipet, when blood is drawn to the 0.5 mark and subsequently diluted to the 11 mark, the dilution is 1/20. The hemoglobin pipets are manufactured to hold 0 02 ml. of blood. Because the blood pipets are carefully calibrated precision instruments they must be given proper care.

Cleaning of the pipets may be performed as follows:

1. Fill the bulb 3 times with distilled water, each time filling through the capillary end, shaking, and emptying through the large bore end.

2. Repeat the above procedure by filling 3 times with 95 per cent alcohol.

3. Then fill the bulb 3 times with ether.

4. *Thoroughly dry the interior of the pipet with a current of dry air.* Air as discharged from the lungs is unsatisfactory since it contains much moisture If a suction apparatus is not available, the pipet may be dried by removing the rubber tubing and shaking in the air. The bead must roll freely and the pipet must be absolutely dry and free from solvents. If any solvent remains in the pipet, protein precipitation will occur when blood is introduced and this may be extremely difficult to remove. The interior of the pipet should always be dry before storing. If moisture remains in the bulb, the bead may adhere to the wall and then be removed only *with difficulty*

In the above procedure, acetone may be substituted for alcohol and ether.

If albuminous material has been allowed to remain in the bulb and cannot be removed by the above method, the pipet should be filled with cleaning solution and allowed to stand overnight

Take all precautions against breaking the point of the pipet. The *slightest nick which enters the bore renders the pipet useless because* of the resulting inaccuracy in dilution.

Hemoglobinometers. A multitude of instruments have been developed and are available for the determination of hemoglobin In no single instrument are the two qualities of accuracy and low

cost incorporated. All instruments, no matter what their cost, must be standardized for the determination of hemoglobin. Even the most expensive spectrophotometer, if not standardized, might be less accurate than a properly standardized, low cost, Sahli hemoglobinometer.

The Sahli hemoglobinometer is the most satisfactory of the low-priced instruments. The square type of Sahli tube with the prismatic-shaped glass standards, although slightly more expensive than other types of Sahli instruments, is the most satisfactory. It is important that the tubes used should be made by the same manufacturer as the standards. The glass standards now produced are reasonably accurate, as shown in table 4. The values in this table were obtained by 100 medical students using 100 different Sahli hemoglobinometers. Hemoglobin was determined on 5 specimens of blood by each student. The sum of the 5 values obtained by each student was divided into the sum of the "correct values." The "correct values" were obtained by the cyanmethemoglobin method and a properly standardized spectrophotometer. The mean hemoglobin value obtained with the Sahli instruments was 6 per cent too high as judged by the distribution curve. However, mean values for the 5 determinations for 86 per cent of the instruments were within ± 10 per cent of the correct values.

The calibrated tube is easily cleaned by brushing with soap and warm water. It is important to keep the glass standards clean and free of dirt and grease.

The Spencer hemoglobinometer (American Optical Company)

TABLE 4—*Standardization of 100 Sahli Hemoglobinometers**

Correct Value / Determined Value	Number of Instruments
1.05—1.07	1
1.02—1.04	4
0.99—1.01	11
0.96—0.98	18
0.93—0.95	37
0.90—0.92	15
0.87—0.89	9
0.84—0.86	1
0.81—0.83	2
0.78—0.80	2

*Purchased in 1956 and 1957.

is more satisfactory than the Sahli hemoglobinometer from the standpoint of accuracy, speed and simplicity. However, its cost is approximately twice that of a Sahli instrument. In this instrument the transmission of light through a thin layer of hemolyzed blood (oxyhemoglobin) is compared with that of a standardized glass wedge with a transmission in the green (wave length, 540 mμ) which closely approximates that of oxyhemoglobin. *Proper light is* obtained by means of a green filter, so that only the intensity of transmitted light of uniform hue is matched in the split field. *A constant and known thickness of the unknown sample of blood* is obtained by filling a chamber of defined depth. Thus, quantitative measurement of the blood sample by the use of a pipet is not *required. The hemoglobin concentration can be determined within* ± 10 per cent (2 C.V.) with this instrument. This is probably the most satisfactory portable, visual hemoglobinometer available *in this country.*

A properly standardized photoelectric instrument is the most accurate, practical method for the routine determination of hemoglobin and should be used whenever possible. With such an instrument the many variables of visual colorimetry are circumvented. There are a great many different instruments available on the market, ranging in price from about one hundred dollars to several thousand dollars. The instruments may be subdivided into two groups: filter-type photometers and spectrophotometers. The filter-type photometers employ monochromatic filters with relatively broad spectral transmission. The spectrophotometers limit the spectrum to very narrow intervals and more truly monochromatic light is obtained. In general, spectrophotometers are to be preferred over filter-type photometers, although hemoglobin determinations can be performed with a sufficient degree of accuracy (± 5 per cent) with even relatively inexpensive instruments of this type. The individual performance of all instruments should be studied with a primary standard, such as copper ammonium sulfate.* In addition, all instruments must be standardized for the determination of hemoglobin (p. 27).

Hematocrit. The hematocrit is a tube of uniform bore (3 mm.)

*For details concerning photometry see D. L. Drabkin, "*Photometry and Spectrophotometry*" in *Medical Physics*, edited by O. Glasser, Chicago, The Year Book Publishers, 1950, vol. 2, p. 1039.

and flat bottom which is calibrated longitudinally into centimeters and millimeters. The numbers on the left of the scale read down from 0 to 10 cm. The numbers on the right read up from 0 to 10 cm. Since no absolute volumetric measurement is required, the volume contained within the tube is of no consequence.

The hematocrit tube is most easily cleaned by inserting a pipet (preferably stainless steel rather than glass) into the bottom of the tube, applying suction (either by a water suction pump or by a syringe fitted to the steel needle) and simultaneously holding the hematocrit tube under a stream of water. The hematocrit is then removed from the current of water, and water from within is withdrawn by suction. If it is then allowed to stand in an inverted position, drying will be completed. When it is necessary to use the tube immediately, rinsing with alcohol and then ether will hasten drying. From time to time the hematocrit should be cleaned with cleaning solution to remove the thin film of coagulated protein which may accumulate on the glass.

Solution Bottles. For students working individually and moving from bedside to bedside and from ward to ward, it is convenient to tape 9 one-ounce bottles together. These bottles can then be filled with buffer, Wright's stain, distilled water, N/10 HCl, reticulocyte stain, alcohol, ether, red cell fluid, and white cell fluid. The first 2 named should each contain a screw cap with a built-in dropper. Corks should be used in the remaining bottles. All bottles and stoppers should be carefully labeled.

Obtaining the Specimen of Blood

Capillary Blood. For small samples such as are needed for the enumeration of red cells, white cells, reticulocytes and platelets and the determination of hemoglobin, puncture of the finger, or, in the case of infants, the plantar surface of the heel provides an adequate specimen with a minimum of difficulty, effort and inconvenience to the patient. For these purposes venous blood has no advantages over capillary blood. Blood smears should always be

made from capillary blood in preference to venous blood to which *an anticoagulant has been added. Blood from an earlobe is less* satisfactory than blood from the finger or heel.

The part to be punctured should not be cyanotic, edematous or cold. If it is *cyanotic it should be immersed in warm water for 5* minutes The side of the finger is less sensitive than the ball of the finger. The skin is cleaned with alcohol and then wiped dry.

The most *satisfactory instrument for capillary puncture is a* disposable stylet.* These are very inexpensive, sharp, and the problem of sterilization is circumvented. Automatic lancets and Bard-Parker blades are unsatisfactory because of the difficulty of adequate sterilization, particularly of the hepatitis virus, and because of the difficulty in keeping them sharp.

A puncture, approximately 3 mm. deep, is made by a simple, quick stroke. The first drop or two of the freely flowing blood is discarded by allowing it to flow off to the side. Slight pressure may be made along the sides of the finger some distance from the wound. Undue pressure in securing drops of blood should be avoided as this will cause dilution of the blood with tissue fluid. If free flowing blood is not obtained, it is better to repeat the puncture. All equipment must be readily available. Speed, dexterity and freely flowing blood are essential for good results.

Venous Blood. The advantage of venous blood is that a number of determinations may be performed and repeated if necessary, at the leisure of the physician, from a single specimen of blood. The blood counts, with the exception of the platelet count, may be made at any time up *to 24 hours after the blood has been drawn,* provided there is no hemolysis evident in the plasma and the bottle has been kept tightly corked and refrigerated at 5 C when not *in use. The sedimentation rate should be performed within 2 hours* and the platelet count within an hour. Venous blood may be used for erythrocyte, reticulocyte, platelet and leukocyte counts, hemo*globin,* sedimentation rate, volume of packed red cells, and icterus index tests.

A 20 or 18 gauge needle with a short bevel should be employed The needle must fit tightly to the syringe If the tip of the syringe

*The "Hemolets" (Scientific Products Div., American Hospital Supply Corp., Evanston, Ill.) or "Sera Sharp" (Abco Dealers Inc., 41 E. 42nd St., New York 17, N. Y.) are satisfactory

is chipped, it must be discarded. All air should be expelled from the syringe before it is used. The needle and syringe must be clean, dry, and sterile.

With the tourniquet in place, a vein is selected, if possible one that is visible, palpable, and well fixed to the surrounding tissue. If a suitable vein cannot be located easily the patient should be instructed to open and close his fist several times, or the site may be gently rubbed. If this fails, it may be necessary to remove the tourniquet and warm the extremity by wrapping it in a warm towel or by soaking it in warm water. It is frequently advantageous in place of a tourniquet to apply a blood pressure cuff equidistant between systolic and diastolic pressure.

A suitable vein having been found and the equipment checked and ready, the area to be punctured is cleansed with 70 per cent alcohol and a tourniquet applied. The skin over the vein is drawn tense by the thumb of the left hand. The syringe is held in the right hand with the bevel of the needle turned up. If the vein is large and well fixed to the surrounding tissues, the skin and the vein may be pierced by one short thrust. If the vein is small or easily movable, it is better to puncture the skin over the vein first and then enter the vein. Only gentle pressure is applied to the plunger of the syringe. Vigorous pull on the plunger of the syringe may cause the vein to collapse, air to enter the syringe, and hemolysis of the blood sample to occur. The tourniquet is removed *before* the needle is withdrawn. The tourniquet should not be applied for more than two minutes since the blood may then be altered by concentration Slow removal of the needle is less painful than sudden removal Gentle pressure is applied over the vein by a pad of clean, dry gauze or cotton ball and the patient is instructed to maintain this pressure for several minutes to prevent bleeding and the development of a subcutaneous hematoma.

Before emptying the syringe into the bottle containing the proper anticoagulant, the needle is removed The contents of the syringe are then gently delivered into the bottle, the plunger of the syringe pulled back so as to prevent "freezing," and the bottle corked. Thorough mixing of the anticoagulant with the blood must be ensured by gently inverting the sample for about one minute The blood must be thoroughly mixed each time before a sample is withdrawn.

It is absolutely essential to use the *proper type and amount of anticoagulant.* Heparin is satisfactory but expensive. Potassium oxalate is unsatisfactory since it causes shrinkage of the red cells. Ammonium oxalate causes the red cells to swell.

The most satisfactory anticoagulant is a mixture of dry ammonium and potassium oxalate. When 6 mg. of ammonium oxalate and 4 mg. of potassium oxalate are used for 5 ml. of blood, no alteration in the volume of packed red cells takes place and the other corpuscular constituents remain unchanged. The volume of blood added to this amount of anticoagulant may be varied from 3.5 to 6.0 ml without appreciable influence on any of the values except the sedimentation rate of the red cells. The anticoagulant is used dry to prevent dilution of the blood. This mixture is referred to as the *double oxalate mixture* and its preparation is as follows.

Ammonium oxalate 1.2 Gm.
Potassium oxalate 0.8 Gm
Neutral formaldehyde (38 per cent U.S.P.) 1 ml
Distilled water to make 100 ml.

From a buret, 0.5 ml. of the above solution is measured into a series of bottles (discarded penicillin bottles with rubber or cork caps are satisfactory). The bottles are allowed to dry at room temperature or in an oven at not more than 60 C. Each bottle then contains 6 mg. of ammonium oxalate and 4 mg. of potassium oxalate, the proper amount for 5 ml. of blood. These bottles may be stored indefinitely without deterioration of the anticoagulant.

Ethylenediamine tetraacetate ("Sequestrene," "Versene") is a very suitable anticoagulant for blood and is relatively inexpensive. The dipotassium salt is more soluble than the disodium salt and is, therefore, more satisfactory for this purpose.* This anticoagulant has the advantage over the "double oxalate mixture" in that platelets are better preserved in it than in the oxalate mixture. The appropriate concentration is 1 mg. per 1 ml. of blood. At this concentration coagulation is effectively prevented and no change in cell volume occurs. A number of bottles for the collection of blood may be prepared as follows:

Dipotassium ethylenediami[illegible] 1.0 Gm.
Distilled water to make 100 [illegible]

*The [illegible] can be [illegible] [illegible] Products, Inc., 550 [illegible] [illegible]

From a buret, 0.5 ml. of the above solution is measured into a series of scrupulously clean bottles. The bottles are allowed to dry overnight at room temperature. Each bottle then contains the proper amount of anticoagulant for 5 ml. of blood. These bottles may be stored indefinitely without deterioration of the anticoagulant.

Examination of the Wet Film of Blood

Technic

1. Place a drop of blood the size of a pinhead on a clean cover glass.

2. Place the cover glass on a clean glass slide in such a manner that the drop is spread without bubble formation.

3. Ring the cover glass with vaseline to prevent drying.

4. Study first with low power, then with high-dry and finally with oil-immersion.

Comments

1. The cover glass and the glass slide must be scrupulously clean.

2. The film of blood must be thin with the cells lying flat and not overlapping or in rouleau formation.

Interpretation

A. Erythrocytes

1. *Color.* The erythrocytes appear greenish-yellow. The intensity of color depends upon the amount of hemoglobin present.

2. *Shape.* The cells are round with a biconcavity, the apparent degree of which depends upon the hemoglobin content. Variations in the shape of the cells are known as *poikilocytosis.*

3. *Size.* The diameter of normal cells is about 7.5 microns. Normally there is only the slightest variation in size. Noticeable variation in size is known as *anisocytosis.* Large cells with a diameter greater than 9 microns are called *macrocytes.* Small cells with a diameter less than 6 microns are called *microcytes.*

being filled. When the bulb is almost full the pipet should be raised to the vertical position and the level of the fluid is drawn slowly to exactly the 101 mark. *The pipet is then shaken immediately* for about 30 seconds to facilitate the initial mixing. For this purpose the pipet may be held loosely in one hand while the attached rubber tubing is revolved between the thumb and forefinger of the other hand. If blood is being obtained from the finger, the manipulations up to this point must be carried out rapidly, otherwise coagulation is likely to begin before the blood is mixed with the diluting fluid and the cells will be clumped in the preparation, thus introducing a gross error.

4. Repeat the above procedures with a second pipet.
5. Shake the pipets for approximately 3 minutes.*

The pipets can conveniently be held between the thumb and second finger. Shaking should be done in several directions rather than in one direction only. When the shaking has been completed, proceed immediately to the next step. If the pipet is allowed to stand for any period of time (even one minute), the contents of the pipet must be remixed for 3 minutes.

6. Expel and discard the first 4 drops from each pipet. This is done in order to remove the fluid in the capillary portion of the pipet which has not come in contact with the blood.
7. Load the counting chambers of the hemocytometer.

Fill one chamber from each pipet. With the cover glass on the chambers over the ruled platforms allow a drop of the diluted blood to run by capillary attraction under the cover glass without any forcing. If this does not occur, the counting chamber or cover glass is dirty and both should be washed in water, dipped in alcohol and dried free of lint. In placing the diluted blood in the counting chambers, the tubing should be kept in the mouth so that the flow can be controlled. The pipet should be held as one holds a pencil, with the tip at the edge of the cover glass. The size of the drop must be gauged by practice. It must be sufficiently large to cover the whole platform and yet not large enough to run into the moat. No bubbles are permissible. If too much has been allowed to run under the cover glass, do not try to save this preparation or to fill

* A pipet rotor such as the Bryan Garrey (A. S. Aloe Co., St. Louis, Mo.) may be used.

the other side of the counting chamber. It is best to clean the count-
ing chambers and start again. If only a slight excess of fluid is left
on the edge of the chamber (not under the cover glass or in the

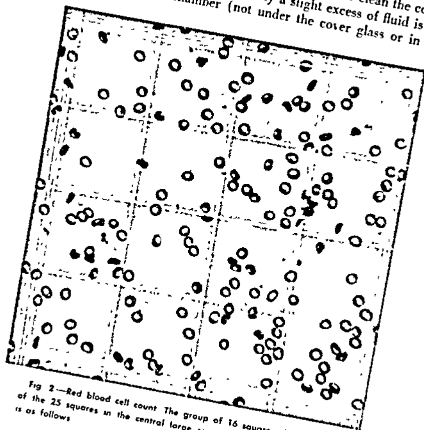


Fig 2—Red blood cell count The group of 16 squares shown is one of the 25 squares in the central large square The accumulative count is as follows

8 → 15 → 29 → 35

69 ← 63 ← 55 ← 43

↓ (69 → 80) ; ↓ (35 → 43)

80 → 90 → 98 → 104

146 ← 137 ← 130 ← 117

↓ (104 → 117)

Considerable skill is required in order to perform accurate red cell counts. This is obtained only through practice. In addition, there is one possible large error (inherent error of distribution) which cannot be eliminated or controlled even in the hands of the most highly skilled. Accurate red cell counts are essential since they are used in the calculation of red cell indices. Because of these facts the following set of rules must be adhered to strictly:

1. Accurately calibrated equipment must be used.
2. All sampling and technical errors must be reduced to a minimum.
3. Two pipets and two chambers must be used.
4. Between 400 and 600 cells must be counted in each chamber.
5. The inherent error of distribution of the cells in the chamber must be estimated.

The accuracy of students performing a red cell count for the sixth time in their experience is shown in table 6. There were 50 students in each class. The red cell counts were performed by a given class on a single specimen of blood. The technic recommended above was used.

TABLE 6—*Variation in Red Cell Count As Performed By Students*

Class	Mean $x10^6/mm^3$	Range $x10^6/mm^3$	2 S D	2 C.V %
1	3 77	3.2 — 4 4	0.52	13.8
2	4.56	3.5 — 5 3	0.51	11.8
3	3.58	3 1 — 4 1	0 46	12.9

S D. refers to standard deviation.
C.V refers to coefficient of variation

Estimation of the Inherent Error of Distribution of the Cells in the Chamber

1. Record the number of cells counted in each chamber (A and B).
2. Obtain the numerical difference C between A and B
3. Calculate two standard deviations by [illegible] wing formula

2 $\sqrt{A +}$ [illegible]

4. If C is [illegible] 2 [illegible] its are [illegible] If C is greater than 2 S.D. [illegible] a [illegible]

5. If the counts are repeated, the average for all 4 chamber counts is taken as the value for the calculation of the red cell count. This is done even though the difference between the third and fourth counts may exceed two standard deviations.

Example: In the first chamber (A) 430 cells were counted.
In the second chamber (B) 470 cells were counted.

$$470 - 430 = 40 \text{ (C)}$$

$$2 \text{ S.D.} = 2\sqrt{430 + 470} = 2\sqrt{900} = 2 \times 30 = 60$$

The difference (C) between the counts is less than 2 S.D. and the counts are acceptable

TABLE 7—*Square Roots*

n	√n	n	√n	n	√n	n	√n	n	√n
300	17.3	400	20.0	800	28.3	1200	34.6	1600	40.0
10	17.6	25	20.6	25	28.7	25	35.0	25	40.3
20	17.9	50	21.2	50	29.2	50	35.4	50	40.6
30	18.2	75	21.8	75	29.6	75	35.7	75	40.9
40	18.4	500	22.4	900	30.0	1300	36.0	1700	41.2
50	18.7	25	22.9	25	30.4	25	36.4	25	41.5
60	19.0	50	23.5	50	30.8	50	36.7	50	41.8
70	19.2	75	24.0	75	31.2	75	37.0	75	42.1
80	19.5	600	24.5	1000	31.6	1400	37.4	1800	42.4
90	19.7	25	25.0	25	32.0	25	37.7	25	42.7
		50	25.5	50	32.4	50	38.1	50	43.0
		75	26.0	75	32.8	75	38.4	75	43.3
		700	26.5	1100	33.2	1500	38.7	1900	43.6
		25	26.9	25	33.5	25	39.0	25	43.9
		50	27.4	50	33.9	50	39.4	50	44.2
		75	27.8	75	34.3	75	39.7	75	44.4

Calculation

The volume of diluted blood in one group of 16 squares is 0.004 mm^3 The volume of diluted blood counted in the 5 groups of 16 squares is 0.004×5 or 0.02 mm^3 To convert to the number of cells in 1 mm^3 of diluted blood, this figure is multiplied by 50 The number of cells in 1 $mm.^3$ must be multiplied by 200 to correct for the initial dilution of 1/200. The final multiplication is then

50×200 or 10,000. In actual practice a decimal point is placed two digits to the left of the number of cells counted in each chamber and the results are expressed in number of cells $\times 10^6$ per mm.[3] The mean of the two or four counts is recorded.

If less than 400 cells are counted in the 5 groups of 16 squares, it is necessary to count additional groups. The calculation then becomes

$$\frac{\text{No. Cells Counted} \times \text{Dilution}}{0.001 \times \text{No. of Groups Counted}}$$

An alternate procedure is to fill the red cell pipet to the 1.0 mark in place of the 0.5 mark, dilute to the 101 mark and count the standard 5 groups of 16 squares. The number of cells counted is then multiplied by 5000 rather than 10,000.

In polycythemia, the number of cells in the counting chamber is too large to permit accurate enumeration if the dilution is 1/200. In this event draw blood to the 0.3 mark of the red cell pipet, dilute to the 101 mark and count the standard 5 groups of 16 squares. The number of cells counted is then multiplied by 10,000 and the result multiplied by 5/3 since only 0.3 part rather than 0.5 part was taken.

Indications for a Red Cell Count

Of the three methods available for the detection of anemia in the laboratory, namely, the red cell count, determination of hemoglobin and the volume of packed red cells, the enumeration of the red cells is the least accurate and the most difficult and time consuming to perform (table 8) Therefore, a red cell count should never be used as a screening procedure for anemia. The only indication for a red cell count is when it is of interest to calculate the red cell indices.

Reagents

Gower's solution

Sodium sulfate 12.5 Gm.
Acetic acid 33.3 ml.
Distilled water to make 100 ml.

TABLE 8.—*Comparison of the Accuracy of the Three Laboratory Methods of Detecting Anemia*

	Minimal Error (2 C.V.) %	Usual Error (2 C.V.) %	Usual Error Limits	True Value
Red cell count (10^6/mm.^{3})	±11	±30*	3.5– 6.5	5.00
Hemoglobin† (Gm. %)	±2	±5	14.3–15.7	15.00
V.P.R.C.‡ (ml./100 ml.)	±1	±1	49.5–50.5	50.00

*One pipet, one chamber and technical error.
†*Photoelectric colorimeter.*
‡V.P.R.C. refers to volume of packed red cells.

Hayem's solution
Sodium sulfate 2.5 Gm.
Sodium chloride 0.5 Gm.
Mercuric chloride 0.25 Gm.
Distilled water to make 100 ml.

For best results this solution should not be more than 3 weeks old.

REFERENCES

1. BERKSON, J., MAGATH, T. B., AND HURN, M.: The error of estimate of the blood cell count as made with the hemacytometer. Am. J. Physiol. *128*, 309, 1940.
2. BIGGS, R. AND MACMILLAN, R. L.: The error of the red cell count. J. Clin. Path. *1*: 288, 1948.

Determination of Hemoglobin

Sahli Method

Technic

1. Fill the calibrated tube to the 2 Gm. mark with 0.1 N HCl. This is done with a dropper. The amount need only be approxi-

mate (within one drop). It is essential that the acid be 0.1 N since lower concentrations fail to produce acid hematin.

2. Fill the pipet with blood to the 0 02 ml. mark.

Care must be taken to fill the pipet exactly to the mark. If a slight excess (2 mm) is taken in, it may be removed by touching the point of the pipet lightly with a finger or cloth. If a great excess of blood is drawn into the pipet, it must be discharged, the pipet cleaned and dried, and the procedure begun again.

3. Wipe off blood adhering to the outside of the pipet.

4. Add the contents of the pipet to the acid in the tube.

The pipet is carefully introduced into the calibrated tube and passed to the bottom into the HCl With gentle constant agitation of the tube, the blood is slowly expelled, care being taken to form no bubbles. When the blood has been expelled the pipet is rinsed by slowly drawing and discharging the acid hematin solution into and out of the pipet several times; care is again taken to prevent bubble formation. The pipet is then withdrawn part way up into the tube and the outside of the pipet is rinsed with several drops of acid. The washings are allowed to fall to the bottom of the tube. The pipet is then removed, the outside wiped off, the inside of the pipet rinsed twice with distilled water, the contents being expelled into the lower portion of the tube.

5. Mix the acid hematin solution.

With the aid of a glass stirring rod, the contents of the tube are mixed. Do not mix by placing a finger over the open end and inverting the tube.

6 Allow the tube to stand for 10 minutes.

The color of the acid hematin solution increases in intensity with time. In 10 minutes 95 per cent of the color is developed, in 20 minutes 98 per cent, in 40 minutes 99 per cent. In practice it is impractical to wait 20 minutes or more Therefore, readings are always made after 10 minutes and the instrument standardized under these conditions.

7. *Dilute the hematin solution until the color matches that of the standard brown glass.*

Add distilled water drop-wise with stirring each time (use glass rod) until the color matches that of the standard brown glass. It is best to employ consistently one type of light. Readings are best made in daylight, but since this is not always available nor of the same

intensity, the preferred procedure is to make the readings with the blue filtered illumination from a substage lamp. The lamp should always be placed in the same position in relation to the hemoglobinometer.

8 Read directly the concentration of hemoglobin in grams per cent.

The bottom of the meniscus of the hematin solution is the level for reading. The scale showing the per cent of normal hemoglobin is disregarded since the amount of hemoglobin varies in normal individuals with age, sex, climate, and other factors, and therefore, no value can be arbitrarily fixed as the equivalent of 100 per cent hemoglobin.

Sources of Error

A. Sampling errors (See under "Enumeration of Erythrocytes," p. 18).
B. Equipment errors
   1. Pipet error.
   2. Error in calibration of tube.
   3 Error in the standardization of the color of the brown glass
C. Technical errors
   1 Unclean or wet pipet.
   2. Improper filling of the pipet.
   3 Blood adhering to the outside of the pipet.
   4 Improper mixing of the blood with the acid.
   5 Unclean tube or standards.
   6. Errors in visual colorimetry.
   7 Variable rate and degree of color development of acid hematin.
   8 Variation in the type and intensity of the light source.

Standardization of the procedure is essential The same pipet, tube, and color standards must be used each time. The time interval allowed for the development of color must remain constant. The light source must remain reasonably constant. Technical errors must be reduced to a minimum Finally, the hemoglobinometer and pipet must be standardized against solutions of known hemoglobin con-

centration with the technic and conditions employed. The accuracy of students determining hemoglobin for the sixth time in their experience is shown in table 9. There were 50 students in each class.

TABLE 9—*Variation in the Determination of Hemoglobin (Sahli Method) as Performed by Students*

Class	Mean Gm./100 ml	Range Gm./100 ml	2.S.D.	2.C.V. %
1	11.6	10.4 — 13.6	1.26	10.8
2	15.4	13.8 — 17.4	1.36	8.8
3	10.9	9.8 — 12.2	0.98	9.0

S.D. refers to standard deviation
C.V. refers to coefficient of variation.

Hemoglobin was determined by a given class on a single specimen of blood. The technic recommended above was followed. A "correction factor" was used for those hemoglobinometers with a "correction factor" greater than ± 10 per cent.

Standardization of the Hemoglobinometer

After the student has gained sufficient practice to have mastered the technic and has standardized the conditions of the technic, a record should be kept of the values obtained on each sample of blood and of the values given him which have been obtained by the use of a well standardized photoelectric instrument. The values obtained by the use of the Sahli and the photoelectric instrument are each totaled. *The "photoelectric values" are divided by the "Sahli values."* If the correction factor obtained is greater than 1.1 or less than 0.9, all subsequent values are multiplied by the factor in order to obtain the corrected values. If the corrected factor is between 0.9 and 1.1, no correction is needed.

Table 10 is supplied so that the standardization of the instrument and the correction factor may be recorded permanently.

Reagent

The 0.1 N hydrochloric acid need not be accurately standardized. It is adequate to dilute 1 ml. of concentrated hydrochloric acid to 100 ml. with distilled water.

TABLE 10.—*Standardization of Sahli Hemoglobinometer*

Sample Number	Determined Value Gm %	Correct Value Gm. %
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
Sum		

$$\text{Correction factor} = \frac{\text{Sum of Correct Values}}{\text{Sum of Determined Values}}$$

Photometric Method (Cyanmethemoglobin)

Photometers are now available in most laboratories With such an instrument it is possible to determine hemoglobin with an accuracy of ± 5 per cent or even ± 2 per cent. Unfortunately, this degree of accuracy has been achieved infrequently in practice in the past. The most important reason for this is that a simple and satisfactory method for standardizing the instrument for the analysis of hemoglobin has not been available "Precalibration" by the manufacturer is frequently more misleading than helpful and cannot be relied upon All instruments must be standardized under the conditions of operation Furthermore, the standardization must be checked at frequent intervals.

An exhaustive study of the problem of finding a suitable, stable,

readily available standard *for hemoglobinometry has been undertaken* by the National Research Council. As a result of this study the Council has concluded that solutions of cyanmethemoglobin are suitable standards for this purpose. Such standards are now readily available commercially from several different sources. Therefore, the applications of these standards will be described in detail.

Cyanmethemoglobin solutions were chosen as a standard for distribution by the National Research Council for the following reasons: (1) the solutions can be prepared simply and inexpensively from crystalline hemoglobin; (2) solutions of this pigment are stable for at least 6 months when preserved at refrigerator temperatures; (3) they can be accurately standardized; and (4) the absorption band of cyanmethemoglobin in the region of 540 mμ is broad rather than sharp, so that its solutions are suitable for *use in filter-type photometers as well as in narrow band spectrophotometers*.

The solutions of cyanmethemoglobin are standardized on the basis that the extinction coefficient of one milligram atom of iron ($c = 1$ mg. atom of iron per liter; $d = 1$ cm.) in the form of cyanmethemoglobin at a wave length of 540 mμ is 11.5. For the purpose of converting the values of hemoglobin from millimoles/liter to mg./100 milliliters, a factor of 1652 is used. This factor is based on an equivalent molecular weight for hemoglobin of 16,520 per atom of iron. In accordance with the decision of the International Union of Pure and Applied Chemistry, based on currently available analysis of crystalline hemoglobin, the iron content of hemoglobin is accepted as 0.338 per cent.

It is possible to standardize the instrument with the cyanmethemoglobin standards and then determine hemoglobin routinely by *a procedure other than the cyanmethemoglobin method* However, the routine determination of hemoglobin as cyanmethemoglobin is recommended for the following reasons: (1) the standardization of other methods of analysis involves additional steps and may lead to some loss of accuracy, (2) *the cyanmethemoglobin method* measures not only oxyhemoglobin but also carbon monoxide hemoglobin and methemoglobin, (3) the cyanmethemoglobin method is simple, and (4) with filter-type photometers the single relatively

broad band of cyanmethemoglobin in the green spectral region has a distinct advantage. A possible disadvantage of the cyanmethemoglobin method is that it involves the use of a solution of cyanide. However, there should be no reluctance to employ this reagent since it contains only 50 mg. of potassium cyanide per liter. The lethal dose of this solution for man approaches four liters. Therefore, it seems that the proper handling of this reagent constitutes a negligible hazard.

Calibration of the Photometer

The instrument should be carefully calibrated for photometry according to the instructions provided by the manufacturer. The method varies with the type and make of instrument. The meter-reading function should be checked. If the instrument is a spectrophotometer the wave length setting should be checked and adjusted if necessary. Some instruments are provided with factory-calibrated standard filters for this purpose. Others are not provided with such a filter and the use of a solution with a known absorption maximum is recommended.

In addition to the cyanmethemoglobin standards it is desirable to have an independent "photometric" standard to serve as a check on the performance of the instrument and on the stability of the cyanmethemoglobin solutions. For example, if the photometric readings of the standard should change appreciably from one reading to another, and if an independent "photometric" standard has been used, then it can be ascertained if the change in the value is due to a change in the instrument or due to a change in the hemoglobin standard.

Solutions of copper ammonium sulfate are very satisfactory as independent "photometric" standards. An appropriate solution may be prepared by dissolving about 1.5 Gm. of copper sulfate $.5H_2O$ in 500 ml. of 2 M ammonium hydroxide. Two molar ammonium hydroxide is prepared by diluting 136 ml. of concentrated (27 to 29 per cent) reagent grade ammonium hydroxide to one liter with distilled water. The copper standard need not be exact since it is used only to determine a change in the reading of the instrument.

The copper ammonium sulfate solution will remain constant for a period of a year when stored in tightly stoppered pyrex flasks.

Matching the Cuvettes

A. Round Cuvettes

1. Clean and dry several dozen cuvettes, taking care that *the outside as well as the inside is clean.*

2. Examine each cuvette for scratches or flaws and discard those which are unsatisfactory.

3. Fill each cuvette with a solution of hemolyzed blood which is prepared by adding 0.2 ml. of whole blood to 50 ml. of distilled water.

4. Set the photometer at the 540 mμ band or put the appropriate filter in place.

5 Set a water *blank* at *100 on the transmittance scale* (T) *or zero on the density scale* (D). *Check this setting at suitable intervals.*

6. Place a filled cuvette in the well and rotate slowly, noting the slight movement of the beam. At the midpoint of the beam's swing, mark the cuvette with a diamond point pencil with reference to some mark on *the housing of the well.*

7. Place a second cuvette in the well and rotate it until the reading of the galvanometer corresponds with the reading at which the first tube was marked. Mark each succeeding tube in the same manner.

8. Precautions should be taken to prevent scratching the cuvettes. Wood or coated wire test tube racks are to be preferred over uncoated wire racks.

B. Square Cuvettes

1. Follow instructions 1 through 5 above.

2. Place each cuvette in the well and record the values. If inexpensive cuvettes are being used, it should be possible to select from a large lot those with identical readings and reserve them for hemoglobinometry If expensive cuvettes are being used, it will be necessary to determine the correction factor for each cell and apply this correction factor to all subsequent determinations and calculations.

Dilution of the Concentrated Standard

Most commercially prepared standards contain approximately 60 mg. of cyanmethemoglobin/100 ml. The exact concentration is stated on the label. At least three different concentrations should be used in the preparation of a standard curve. Therefore, it is necessary to make at least two different dilutions of the concentrated standard. Appropriate concentrations to read for the standard curve are about 60, 40 and 20 mg. of cyanmethemoglobin/100 ml. These concentrations are equivalent to 15, 10 and 5 Gm. of hemoglobin/100 ml when blood is diluted 1:251.

A convenient and accurate way to prepare the higher dilutions is as follows:

1. Set up 3 large, clean test tubes.
2. By the use of a clean 5 ml. volumetric pipet transfer 5 ml. of the concentrated standard (circa 60 mg./100 ml.) to each of the 3 tubes.
3. By the use of the same pipet transfer an additional 5 ml. of the concentrated standard into tube 2.
4. Carefully rinse the pipet 5 times with the diluent solution.
5. Pipet 5 ml. of the diluent solution into tube 2 and 10 ml. into tube 3.
6. After appropriate mixing, transfer 5 ml of the contents of each tube into each of 3 matched cuvettes
7. The concentration of cyanmethemoglobin in tube 1 is the same as that stated on the standard solution. The concentration in tube 2 is two-thirds that of the concentrated standard. The concentration of cyanmethemoglobin in tube 3 is one-third that of the standard solution By making the dilutions as outlined above with a single pipet, the problem of the exact volume of the pipet is avoided.
8. An exception to this procedure must be made for the Evelyn photometer The most concentrated of the above 3 standards cannot be read because hemoglobinometry in this instrument is carried out at a dilution of 0.02 ml of blood in 10 ml. of diluent, representing 501-fold dilution. In this case, it is recommended that 5 ml. of the concentrated standard be pipetted into each of 3 test tubes. By the use of the same pipet transfer 5 ml. of diluent to tube 1,

10 ml. to tube 2, and 20 ml. to tube 3. The concentration of cyanmethemoglobin in tube 1 is then one-half that of the concentrated standard, in tube 2 one-third, and in tube 3 one-fifth that of the concentrated standard.

Care of the Standards

The standards are stable for at least 6 months when maintained under proper conditions. The solutions, as obtained, are free of bacterial contamination. They are bacteriostatic though not bactericidal and should remain free of contamination if properly handled. The most satisfactory method of handling is to transfer them to clean, matched cuvettes and seal them permanently. Rubber or cork stoppers are less satisfactory. Care should be taken to prevent scratching of the cuvettes. When not in use, the standards should be stored in a refrigerator (5 C.) in the dark, but not frozen. Should they become turbid, they are obviously not satisfactory for photometry.

The standards should be warmed to room temperature before use. *This is extremely important because when the solution is cold* and is then heated by the light source of the photometer, condensation on the optical surfaces of the cuvette will occur. This may cause a considerable error. Also, when the cold solution is warmed by the light source, bubbles may form in the solution Even small bubbles on the optical surfaces of the cuvette may give rise to an appreciable error. The cuvettes should be wiped free of outside *moisture, grease and lint before use.*

Every 6 months a new concentrated standard solution should be purchased and the old solutions discarded.

Calibration of the Instrument With the Cyanmethemoglobin Standards

1. The measurements are made at wave *length of 510 mμ If the instrument is a spectrophotometer* set the wave length selector at 510 mμ. If a filter-type photometer is used, the appropriate filter must be in place (Evelyn, 510 filter; Leitz Photometer, filter disk 550; Fisher Clinical Electrophotometer, 525 filter, Klett-Summerson Photoelectric Colorimeter, green (No. 51) filter)

2. Turn on the instrument; allow it to warm up. The operation of each make of clinical photometer is somewhat different. Instructions concerning operation come with each instrument.

3. The absorption of light by the diluent solution at 540 mμ is negligible. Therefore, either the diluent solution or distilled water may be used as a blank.

4. Set the blank at 100 on the per cent transmittance (T) scale or at zero on the density (D) scale.

5. Place each of the three standard tubes in the cuvette well and read the values from the scale.*

6. It is difficult to state arbitrarily the frequency with which the standardization should be checked. Perhaps, until the operator becomes acquainted with the constancy of the instrument under his particular conditions, it is advisable to make a check (this takes only about 5 minutes) every time a determination is performed. If after considerable experience it is found that the standardization is constant over prolonged periods, less frequent checking may be necessary. In general, it is recommended that a regular schedule of weekly checking with the standards should be instituted. More frequent checking would be within the judgment of the operator.

Preparation of a Standard Curve and Table

1. The concentrations of the standards refer to the concentration (mg /100 ml) of cyanmethemoglobin in the solutions. To obtain the values for the amount of hemoglobin in 100 ml. of undiluted blood, these values are multiplied by the dilution factor and the product is divided by 1000 If 0.02 ml. of blood is added to 5 ml. of diluent solution, the dilution factor is 251. If 0 02 ml. of blood is added to 10 ml of diluent solution (Evelyn instrument), the dilution factor is 501.

*To span the light beam of the Coleman, Jr Spectrophotometer there must be at least 6 ml of solution in the 19 mm. cuvette The accurate measurement of 6 ml is cumbersome because transfer pipets are not available in that size and serologic pipets are inadequate Therefore, it is recommended that the instrument be slightly modified to permit accurate readings with a smaller volume of solution A 5 mm slice should be cut from the top of a no 1 rubber stopper and dropped into the cuvette adapter so that it lies flat on the bottom This elevates the cuvette sufficiently that 5 ml of solution span the light beam

For example, if the concentration of the cyanmethemoglobin standard is exactly 58.9 mg. per cent and the dilution 251, then

$$\frac{58.9 \times 251}{1000} = 14.78 \text{ Gm. of hemoglobin/100 ml.}$$

2. If the meter readings are in terms of per cent transmission (T), the values of the standards are plotted on single-cycle semilogarithmic paper with the per cent transmission on the ordinate (logarithmic scale) and the hemoglobin in grams per cent on the abscissa (X axis or bottom axis). A line connecting the three points should pass through or very near T per cent = 100. This is the standard curve. The T per cent values for unknown bloods may be translated into values of hemoglobin by reference to this curve. When large numbers of measurements are made it may be more convenient to construct a table of hemoglobin values for every possible T per cent reading.

3. If the meter readings are in terms of density (D) the values are plotted on linear graph paper with the density values on the ordinate (Y axis or left border) and the hemoglobin in grams per cent on the abscissa (X axis or bottom axis). A line connecting the three points should pass through or very near zero The D values for unknown bloods may be translated into values for hemoglobin by reference to this curve, or a table of hemoglobin values for every possible D reading may be prepared from it.

Determination of Hemoglobin as Cyanmethemoglobin

1. Measure exactly 5 ml. of the diluent solution into a clean, dry cuvette or test tube. When square cuvettes are used it is usually more convenient and satisfactory to mix the blood and diluent in a clean, dry test tube and transfer the solution to the cuvette at the time it is placed in the instrument. The 5 ml. volumetric transfer pipet should be within the tolerance set by the U. S. Bureau of Standards. The pipet should be filled by the use of a suction bulb.* When many determinations are performed it is more convenient to use an automatic pipet or buret.†

*"Volupettor," 5 ml. Standard Scientific Supply Corp., 34 West 4th St., New York 12, N. Y., is satisfactory.

†The Blue Line "Exax," automatic, overflow pipet is satisfactory. It can be connected to a large reservoir bottle and to an overflow bottle. This pipet can be purchased from A. H. Thomas Company, Philadelphia, Pa.

2. Transfer exactly 0.02 ml. (Sahli pipet) of blood into the diluent solution. Care must be taken to fill the pipet exactly to the mark. If a slight excess (2 mm.) is taken in, it may be removed by touching the point of the pipet lightly with a finger of cloth. If a great excess of blood is drawn into the pipet, it must be discharged, the pipet cleaned and dried, and the procedure begun again.

3. Wipe off blood adhering to the outside of the pipet.

4. Rinse the pipet three times with the diluent in the cuvette (test tube). Care should be taken to prevent the formation of air bubbles in the solution.

5. Mix the blood and the diluent thoroughly by rotating the tube.

6. Allow 10 minutes for the cyanmethemoglobin to form.

7. Measure 5 ml. of diluent solution or distilled water into a second cuvette to serve as a blank.

8. Wipe the outside of the cuvettes clean.

9. Put the appropriate filter (see p. 32) in place or adjust the wave length scale to 540. Turn on the instrument and allow it to warm up. Determine the T per cent or D value of the unknown in the same manner as described above for standards. From the tandard curve or table obtain the hemoglobin value of the nknown.

termination of Hemoglobin By Methods Other than Cyanmethemoglobin

1. Calibrate the instrument by the use of the cyanmethemoglobin standard as described above and prepare a standard curve.

2. Draw 30 ml. of blood from a normal subject. Place the blood in an Erlenmeyer flask containing 5 drops of a 20 per cent solution of potassium oxalate. Mix well and transfer 2 ml. to each of five small test tubes. Centrifuge the remainder of the blood to obtain the plasma. Add 0.5 ml. of plasma to the second test tube containing the whole blood, 1.0 ml. to the third, 2.0 ml. to the fourth and 4.0 ml. to the fifth.

3. Mix the contents of the tubes well and determine the hemoglobin values for each by the cyanmethemoglobin method.

4. These five samples of blood of known hemoglobin concentration can now be used as "secondary standards." By the chosen method of analysis, determine the T per cent or D values on each.

5. Prepare a standard curve as described above by plotting the T per cent (semilogarithmic paper) values or the D values (linear paper) on the ordinate and the hemoglobin values in grams per cent on the abscissa. A straight line is then fitted to the points. This line should pass through or very near T per cent = 100 or D = 0.

6. To check the standardization from time to time the procedure can be repeated on a single specimen of blood. If the instrument must be restandardized the entire procedure should be repeated.

7. If the analyses are performed by the oxyhemoglobin method, the standardization should be completed within four hours after the blood has been withdrawn, since methemoglobin will form on standing and this pigment is not measured by the oxyhemoglobin method.

Reagent

Diluent Solution (Drabkin's solution)
Sodium bicarbonate 1.0 Gm.
Potassium cyanide 0.05 Gm.
Potassium ferricyanide 0.20 Gm.
Distilled water to make 1000 ml.

The chemicals used should be of reagent grade quality. The solution should be kept in a brown bottle and should be relatively fresh. More than one month's supply should not be prepared at one time. The solution is clear and pale yellow in color. If it becomes turbid it should be discarded.

While the concentration of cyanide in the diluent solution is insignificant, the salt itself is poisonous and should be handled only by highly responsible personnel. If small amounts of cyanide compounds are accidentally spilled during the weighing procedure, the dry powder should be wiped up first with a dry cloth and then with a moist cloth. The cloths should be discarded into a suitable, closed container. Cyanide solutions or powder should not be placed in a sink with acid The solution may be discarded into a sink with the water flowing freely. The solid potassium cyanide should be stored in a locked cupboard

REFERENCES

1. CANNAN, R. K.: Proposal for the distribution of a certified standard for use in hemoglobinometry. Blood 10: 562, 1955.
2. DRABKIN, D. L.: Photometry and spectrophotometry. In GLASSER, O., Ed.: Medical Physics Chicago, Year Book Publishers, 1950, vol 2, p 1039.
3. CROSBY, W. H., MUNN, J. I., AND FURTH, F. W.: Standardizing a method for clinical hemoglobinometry U. S Armed Forces Med. J. 5: 693, 1954.

Erythrocyte Sedimentation Test

Technic

1. Fill the hematocrit to the 10 mark with venous blood. This can be done by passing a filled hematocrit pipet to the bottom of the hematocrit. The blood is gradually expelled as the pipet is raised. Care should be taken to produce no bubbles of air in the tube.
2. Set the tube in a vertical position in an appropriate rack.
3. At the end of 1 hour record the number of mm which the corpuscles have fallen.

Precautions and Sources of Error

1. Care must be taken to insure adequate mixing of the specimen.
2. The standard quantity of the double oxalate anticoagulant must be used Higher concentrations of anticoagulant tend to delay sedimentation
3. The collected blood should be used for the determination of the sedimentation rate as soon after it is drawn as possible Delay beyond 2 hours may be associated with delayed sedimentation rate.
4. The hematocrit tube must be clean, dry and free of all alcohol and ether.
5. There is an error due to variation in the diameter (2.75 to 3 25 mm) of the hematocrit.
6. The hematocrit must be kept in an exactly vertical position

during the sedimentation of the blood corpuscles, for when the instrument stands at an angle of even 3 degrees from the vertical, significant acceleration (30 per cent) of the sedimentation takes place.

7. The *sedimentation rate increases with increasing temperature.* Ideally, the test should be carried out at constant temperature, preferably 20 C. However, because the sedimentation rate is only slightly influenced by temperatures between 22 and 27 C., and because the test is crude at best, this refinement is usually unnecessary. *If the room temperature fluctuates greatly above or below* 22 to 27 C., or if more accurate sedimentation rates are required, the temperature should be controlled. This can be done by immersing the tubes in a constant temperature bath. Or, by the use of a temperature correction chart, the observed rate at given temperature *may be corrected to 20 C. (For such a chart see Am. J. M. Sc. 212:* 207, 1946.)

8. The quantity of corpuscles in suspension influences the stability of the suspension. When anemia is present, sedimentation velocity will be greater, and when polycythemia is present, it will *be slower than in blood containing a normal quantity of corpuscles.* It has been the practice in the past to "correct" the sedimentation rate according to the volume of packed red cells by the use of a "correction" chart. However, such "correction" charts are frequently misleading. Because of this, and because the sedimentation *rate is of little importance in patients with anemia, it is of little* value to "correct" the sedimentation rate according to the degree of anemia. It is important, however, to recognize that anemia per se will cause an increased sedimentation rate, and that polycythemia will cause a decreased rate of sedimentation

9. *A final note of precaution is necessary.* It must be recognized that the sedimentation test is subject to many errors and is a crude approximation. Balanced clinical judgment is necessary in its interpretation. When this attribute is applied to the values obtained in the laboratory, the test may be extremely useful When this attribute is lacking, the test is more often misleading than helpful.

REFERENCE

WINTROBE, M M AND LANDSBERG J W A standardized technique for the blood sedimentation test Am J M Sc *189* 102, 1935

Volume of Packed Red Cells

Technic

1. After the sedimentation test has been performed the hematocrit is centrifuged for 30 minutes at a relative centrifugal force (R C F.) of 2260 × gravity. If the sedimentation rate is not desired, the hematocrit may be centrifuged immediately. However, a more distinct separation of the red cell layer from the leukocytes and platelets will be obtained if the blood is first allowed to sediment.

2. The volume of packed red cells (V.P.R C.) may be read directly from the numbers on the right side of the scale. This scale is divided into centimeters and millimeters and the level of packed red cells can be estimated to the nearest one-half mm. The level at which the packed red corpuscles are found, if multiplied by 10, will give the volume per 100 ml. of blood, providing the tube was filled to "10" (which is to the right of the scale and is opposite the 0 mark at the left of the scale) If the hematocrit is not filled to "10," then the volume of packed red cells, as read, is divided by the reading at the meniscus made by the blood plasma prior to centrifugation, or after centrifugation if the tube has been capped to avoid evaporation

At the uppermost portion of the packed red cells, immediately adjacent to the reddish-gray layer of packed white corpuscles, a narrow black band composed of reduced oxyhemoglobin will be seen The reading of "volume of packed red cells" is made at the uppermost portion of the black line.

Sources of Error

1. Sampling errors (see under "Enumeration of Erythrocytes," p 18 and the section on "Obtaining the Blood Specimen," p. 10) These are avoidable errors.

2. The proper type and amount of anticoagulant must be used; namely, 6 mg. of ammonium oxalate and 4 mg. of potassium oxalate or 5 mg of Versene per 5 ml. of whole blood (p 12).

3 The hematocrit must be clean and dry.

4 The variation involved in the reading of the level by different observers is approximately ±0 34 per cent (±2 C.V.). Care must

be taken to avoid misreading the level of packed cells by 5 mm. This is a common and often unrecognized visual error.

5. There is an error due to differences in tubes which is approximately ±0.79 per cent (±2 C.V.).

6. The total error or variation involved in the determination of V.P.R.C. (providing the hematocrit is properly centrifuged) amounts to approximately ±1.13 per cent (±2 C.V.). Compared with the error involved in most laboratory procedures, clinical or otherwise, this error is extremely small. By comparison with the error involved in enumerating erythrocytes (at least ±11 per cent and frequently ±30 per cent) and the error involved in the determination of hemoglobin (at least ±2 per cent [photoelectric] and frequently ±15 [Sahli]), *it is obvious that this is the most accurate procedure available for the detection of anemia or polycythemia* (table 8) And not only is the determination of V.P.R.C. the most accurate method of the three, but also the simplest and the least time consuming to perform. Furthermore, many determinations may be performed simultaneously, and information concerning the sedimentation rate, icterus index and volume of packed leukocytes and platelets may be obtained with the expenditure of little additional effort. To repeat, in order to emphasize this point still further, the determination of V.P.R.C is the procedure of choice in quantitating the degree of anemia or polycythemia. The only indication for enumerating the erythrocytes is when the hematocrit value is abnormal and it is desirable to compute the size of the red corpuscles. If the V.P.R.C is normal, under most circumstances, nothing further need be done

7. Optimal packing of the cells in the hematocrit must occur To insure this it is necessary that a force of 2260 × gravity be exerted on the cells in the tube. This force (relative centrifugal force or R.C.F. expressed in number of times gravity) is dependent not only upon the number of revolutions per minute (R.P.M.) but also upon the radius (r) as measured in cm. from the center of revolution to the bottom of the hematocrit. This relationship is expressed in the following formula:

R.C.F. = 0 ... 1118 (... .P.M.^{2}

From this equation, ... radia ... (r) and the number of revolutions per mi ... l.), ... centrifugal force

(R.C.F.) applied to the red cells can be calculated for any given centrifuge. Substituting in the formula a R.C.F. of 2260 × gravity, the equation may be rewritten

$$R.P.M. = \sqrt{\frac{202116700}{r}}$$

and used to determine the number of revolutions per minute (R.P.M.) a given centrifuge with a radial distance of r cm. must rotate in order to effect optimal packing of the red cells. In actual practice a tachometer is necessary to determine the R.P.M. The R.P.M. stated on the face of the centrifuge cannot be accepted. Most large laboratories are equipped with a standard International (size 2) centrifuge with an attached tachometer. If two hematocrits

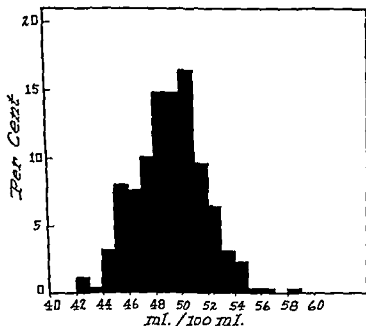


Fig 3—Frequency distribution diagram for the volume of packed red cells in 250, presumably healthy, male, second year medical students residing in Salt Lake City, Utah (altitude 4390 feet) The mean value is 49 ml /100 ml

are filled from the same bottle of blood and one is centrifuged for 30 minutes at 3000 R.P.M. (as determined with the tachometer) in an International (size 2) centrifuge (where r = 22 5 cm. and R.C.F. = 2260 × gravity) and the second hematocrit centrifuged in one's own centrifuge,* the rheostat setting on the small centrifuge which will give the same degree of packing as the hematocrit centrifuged at 2260 × gravity can then be determined. This should be checked from time to time, since as a centrifuge is used, its efficiency diminishes. It should be noted that prolonging the time of centrifugation beyond 30 minutes will not cause greater packing and consequently time cannot be used as a substitute for force (R.C.F.).

REFERENCE

WINTROBE, M. M.: A simple and accurate hematocrit. J. Lab & Clin. Med 15: 287, 1929.

Volume of Packed White Cells and Platelets

Above the deep red layer of packed red corpuscles in the hematocrit, a reddish-gray layer of packed leukocytes and platelets is found. When the platelets are more numerous than usual, and sometimes even when they are present in normal numbers, it is possible to distinguish two portions in the layer above the red corpuscles. Uppermost will be found a cream-colored layer which consists practically entirely of platelets. The reddish-gray layer below this consists almost entirely of leukocytes. Separation of the corpuscles into layers is aided by a period of sedimentation preceding centrifugation.

The thickness of the layer above the packed red corpuscles depends on a number of factors; namely, the number of leukocytes, the kind of leukocytes, and the quantity of platelets. Since lymphocytes are smaller than the cells of the myeloid series, when there

*The Clay Adams angle head, table model centrifuge CT 1002, is relatively inexpensive and is suitable for this purpose

is relative lymphocytosis the layer will be narrower for a given quantity of corpuscles than when the myeloid leukocytes predominate. If the platelets are reduced in number the layer is correspondingly narrower. In cases of leukopenia, and particularly when this is accompanied by thrombocytopenia, the layer of corpuscles above the red cells is barely perceptible. On the other hand, in cases of leukemia with marked leukocytosis, this layer may be even as great as the volume of packed red cells.

When the platelet count is approximately normal, the thickness of this reddish-gray layer may be used as a rough index of the leukocyte count. In normal blood this layer measures 0.5 to 1 mm. in thickness and each 0.1 mm. corresponds approximately to 1000 leukocytes/mm.^{3} When leukocytosis is marked, relatively more packing of leukocytes tends to occur than in normal blood and 0.1 mm corresponds more nearly to 2000 leukocytes per mm.^{3} than to 1000.

Measurement of the corpuscular layer above the layer of packed red cells is not recommended as an alternative to leukocyte or platelet counts. However, it is evident that this can serve as a useful rough guide and is important in routine studies in calling attention to marked alterations in the numbers of these corpuscles.

REFERENCE

WINTROBE, M. M.: Macroscopic examination of the blood. Am. J. M. Sc. 185: 58, 1933

Determination of Icterus Index

Technic

The yellow color of the plasma, as seen in the hematocrit after it has been centrifuged, is compared with that of a series of standard potassium dichromate solutions placed in tubes of glass of the same thickness and bore as the hematocrit. The results are expressed in units. One unit is defined as the color of an aqueous solution

of potassium dichromate containing 0.01 Gm. per 100 ml. Tubes corresponding to 1, 3, 5, 7.5, 10, 25, 50 and 100 units are customary.

Comment

The icterus index is a useful, simple, and rapid test for semiquantitatively estimating the bilirubin content of the plasma. When the icterus index is normal, it can be assumed that there is no *increase in the bilirubin content of the plasma. In the range of* 5 to 25 units each unit is roughly equivalent to 0.1 mg. of bilirubin. In the range of 25 to 50 units this quantitation is less reliable, and in the range from 50 to 100 units it ceases to be even semiquantitative. But if the icterus index is in this high range, it can be assumed that the bilirubin level is markedly elevated. If more accurate information is desired, a quantitative bilirubin estimation should be performed (p. 217).

Other than the fact that the test is only semiquantitative, there are several other limitations. If the blood is hemolyzed or lipemic, the icterus index cannot be read. The test is not specific for bilirubin itself since yellow color of the plasma may be produced by carotene and other xanthochromic pigments and by certain drugs such as atabrine, chlorpromazine and dinitrophenol. However, the occurrence of these interfering substances is rare.

REFERENCE

WINTROBE, M. M.: Macroscopic examination of the blood. Am. J. M. Sc. *185*: 58, 1933.

Calculation of Corpuscular Constants

Technic

1. The *mean corpuscular volume* (M.C.V.) is the volume of the average R.B.C. of a given sample of blood and is determined by dividing the volume of packed red cells (V.P.R.C.), expressed

in ml. per 1000 ml. of blood, by the red cell count (R.B.C.), expressed in millions.

$$\text{M.C.V. in } \mu^3 = \frac{\text{V.P.R.C. (ml./1000 ml. blood)}}{\text{R.B.C. } (10^6/\text{mm.}^3)}$$

Example: A given blood contains 5.1 × 10^6/mm.^{3} R.B.C. and 44 6 ml /100 ml. packed R.B.C.

Then,

$$\text{M C.V.} = \frac{446}{5.1} = 87\mu^3$$

2. The *mean corpuscular hemoglobin* (M.C.H.) is the amount of hemoglobin, by weight, in the average R.B C. of the sample of blood. It is determined by dividing the hemoglobin (Hb) of the blood, expressed in grams per 1000 ml. of blood, by the R.B.C., expressed in millions.

$$\text{M.C.H. in } \mu\mu\text{g.} = \frac{\text{Hb. (Gm./1000 ml. blood)}}{\text{R.B.C. } (10^6/\text{mm.}^3)}$$

Example. R B C. 5.1 × 10^6/mm.^{3}; Hb. 15 2 Gm./100 ml

Then,

$$\text{M.C.H.} = \frac{152}{5\,1} = 30 \text{ micromicrograms } (\mu\mu\text{g.}).$$

3 The *mean corpuscular hemoglobin concentration* (M.C.H.C.) is the proportion of hemoglobin (weight/volume) contained in the average red cell of the sample of blood. It is determined by dividing the hemoglobin, expressed in grams per 100 ml., by the volume of packed red cells, expressed in ml. per 100 ml , and multiplying the result by 100.

$$\text{M C.H C. in } \% = \frac{\text{Hb. (Gm /100 ml. blood)}}{\text{V.P.R.C. (ml./100 ml. blood)}} \times 100$$

Example· Hb 15.2 Gm /100 ml., V P R.C. 44.6 ml /100 ml.

Then,

$$\text{M C.H C.} = \frac{15\,2}{44.6} \times 100 = 34\%.$$

Comments

The accuracy of students determining the corpuscular constants for the sixth time in their experience is recorded in table 11. There were 50 students in each class. The erythrocytes were enumerated by the method described on p. 15. Hemoglobin determinations were performed by the Sahli method (p. 23). A "correction factor" was used for those hemoglobinometers with a "correction factor" greater than ± 10 per cent. The value for the volume of packed red cells was given to each class and was not determined by individual students.

TABLE 11.—*Variation in the Corpuscular Constants as Determined by Students*

	Class	Mean	Range	2 S.D.	2 C.V. %
Mean Corpuscular Volume μ^3	1	92	79 — 107	12.2	13.2
	2	95	81 — 123	15.4	16.2
	3	95	83 — 110	11.2	11.8
Mean Corpuscular Hemoglobin $\mu\mu g.$	1	30	24 — 35	4.8	16.0
	2	34	28 — 41	5.2	15.3
	3	31	27 — 37	5.0	16.2
Mean Corpuscular Hemoglobin Concentration %	1	33	29 — 36	3.6	10.9
	2	35	32 — 37	3.6	10.3
	3	32	30 — 36	3.6	11.2

S.D. refers to standard deviation
C.V. refers to coefficient of variation

The variation in the corpuscular constants performed by three experienced technicians is shown in table 12. Each technician determined the constants four times on the same specimen blood. The counts were performed with different pipets and chambers. Hemoglobin was determined as cyanmethemoglobin by the use of an Evelyn photometer.

It must be pointed out that if the red cell count, hemoglobin, and volume of packed red cells are not determined exactly as

TABLE 12—*The Accuracy of the Corpuscular Constants as Determined by Laboratory Technicians*

Determination	Mean	Range	2 S D	2 C.V. %
Red Cell Count, $x10^6/mm^3$	5 45	4 83 — 6 45[1]	0 94	17.3
Hemoglobin, Gm %	15 6	15 4 — 15 9	0 36	2 3
Mean Corpuscular Volume μ^3	92	77 — 103	15 6	16 9
Mean Corpuscular Hemoglobin $\mu\mu g$	29	24 — 33	1 76	6 1
Mean Corpuscular Hemoglobin Concentration %	31	31 — 32	1 04	3 4

[1]Technician A, 4 83 — 5.21; technician B, 5 23 — 5 52; technician C, 5 73 — 6 45

described, the corpuscular constants will be worthless and misleading. Even when these precautions are taken *it is always necessary to check the appearance of the erythrocytes in the stained film against the values for the corpuscular constants*

When computed from reliable data and checked against the blood smear, the corpuscular constants are extremely useful clinically. From them a morphologic classification has been devised which is not only of assistance diagnostically but also gives important guidance concerning therapy (table 13).

TABLE 13—*Morphologic Classification of Anemia**

Type of Anemia	M C V μ^3	M C H $\mu\mu g$	M C.H C %
Macrocytic	94 — 160	32 — 50	32 — 36
Normocytic	82 — 92	27 — 31	32 — 36
Simple microcytic	72 — 80	21 — 24	30 — 36
Microcytic hypochromic	50 — 80	12 — 29	24 — 30

* From Wintrobe, *Clinical Hematology*, 4th edition, Philadelphia, Lea and Febiger, 1956

REFERENCE

WINTROBE, M. M.: The size and hemoglobin content of the erythrocyte. J. Lab & Clin. Med. *17*: 899, 1932.

TABLE 14—*Normal Erythrocyte and Platelet Values**

Determination	Sex	Mean	Range
Red blood cells, × $10^6/mm^3$	Male	5.4	4.6—6.2
	Female	4.8	4.2—5.4
Hemoglobin, Gm %	Male	16	14—18
	Female	14	12—16
Volume packed red cells, ml/100 ml	Male	47	40—54
	Female	42	37—47
Mean corpuscular volume, μ^3	Both	87	82—92
Mean corpuscular hemoglobin, $\mu\mu g$	Both	29	27—31
Mean corpuscular hemoglobin concentration, %	Both	34	32—36
Reticulocytes, %	Both	1.0	0.5—1.5
Erythrocyte sedimentation rate, mm/hr	Male	4	0—10
	Female	10	0—15
Icterus index units	Both		5—7.5
Platelets, × $10^3/mm^3$	Both	250	200—300
Normal Erythrocyte Values for Salt Lake City†			
Red blood cells, × $10^6/mm^3$	Male	5.8	4.6—6.3
	Female	5.2	4.4—5.8
Hemoglobin, Gm %	Male	17	15—18
	Female	15	13—17
Volume packed red cells, ml/100 ml	Male	50	44—55
	Female	45	39—48

* Sea level

† Altitude 4390 feet

TABLE 15 *The Macrocytic Anemias*

A. Megaloblastic macrocytic anemias
   1. Pernicious anemia (Scandinavia, England, U. S.)*
   2. Sprue, idiopathic steatorrhea (W. W.)
   3. Nontropical nutritional macrocytic anemia (Southeastern U. S.)
   4. Intestinal strictures, blind segments, resections, etc. (W. W.)
   5. Diphyllobothrium latum infestation (Finland)
   6. Megaloblastic anemia of infancy (U. S., Italy)†

7. Tropical macrocytic anemia (India)
8. Megaloblastic anemia of pregnancy (W. W.)†
9. Refractory megaloblastic anemia (Scotland)
10. Achrestic anemia (England)

B Nonmegaloblastic macrocytic anemias
1. Reticulocytosis from any cause such as posthemorrhagic anemia, hemolytic anemias
2. Liver disease†
3. Hypothyroidism†
4. Aplastic anemia†
5. Scurvy†

* Pernicious anemia is distinguished from the other conditions listed in that achlorhydria is always present and neurologic changes may occur.

† The anemia may be normocytic rather than macrocytic.

The geographic distribution given refers to the regions where the incidence is high or where cases have been reported W W refers to world wide.

The first five conditions listed under "A" respond hematologically to either vitamin B_{12} or folic acid Conditions 6 and 7 respond to folic acid but a few cases responding to vitamin B_{12} have been reported The last three diseases listed under "A" respond to folic acid but not to vitamin B_{12}.

N B In practice, the most common cause of "macrocytic anemia" is laboratory error, and this is most often due to errors in red cell counting

TABLE 16—*The Normocytic Anemias*

A Posthemorrhagic anemia
1. Injury of large vessels
2. Coagulation defect
3. Platelet deficiency
4. Abnormality in blood vessels

B Hemolytic anemia (see table 41)
1. Intracorpuscular (Hereditary)
2. Extracorpuscular (Acquired)

C Hypoplastic anemia
1. Idiopathic
2. Secondary

D Associated with chronic disease
1. Renal disease
2. Infections
3. Malignancy
4. Liver disease
5. Hypothyroidism
6. Collagen disorders
7. Rheumatoid arthritis
8. Rheumatic fever

E. Associated with bone marrow invasion by
   1. Metastatic carcinoma
   2. Leukemia
   3. Lymphoma
   4. Multiple myeloma
   5. Myelosclerosis and fibrosis
   6. *Marble bone disease*

F. Associated with splenomegaly
   1. *Idiopathic*
   2. Tuberculosis
   3. Malaria
   4. Kala Azar
   5. Sarcoidosis
   6. Collagen disorders
   7. Obstruction of the splenic vein
   8. *Syphilis*
   9. Lymphoma
   10. Felty's Syndrome
   11. Amyloidosis
   12. Gaucher's disease
   13. Liver disease

G. Associated with pregnancy

TABLE 17.—*Simple Microcytic Anemia**

A. Chronic inflammatory diseases
   1. Lung abscess
   2. Empyema
   3. Bronchiectasis
   4. Subacute bacterial endocarditis
   5. *Pelvic inflammatory diseases*
   6. Chronic osteomyelitis
   7. Tuberculosis
   8. Brucellosis
   9. Meningococcemia
   10. Cellulitis
   11. Chronic fungus infections
   12. Arthritis
   13. Rheumatic fever

B. *Chronic noninflammatory diseases*
   1. Plumbism
   2. Malignancy
   3. Endocrine disorders (hypoadrenalism, hypopituitarism)
   4. Hereditary spherocytosis
   5. Hemoglobinopathies (Hb C, D, F diseases)

* The anemia associated with all of the conditions listed is frequently normocytic rather than microcytic

TABLE 18.—*Microcytic Hypochromic Anemia*

A. Iron deficiency anemia
B. Hemoglobinopathies (see table 13)
   1. Hemoglobin C disease

2. Hemoglobin D disease
3 Thalassemia and Thalassemic syndromes

TABLE 19 —*Causes of Pancytopenia*

A Hypoplastic (aplastic) anemia

B Aleukemic leukemia

C. Pernicious anemia

D. Diseases associated with bone marrow invasion (myelophthisic anemia)
1 Metastatic carcinoma
2. Lymphoma
3 Multiple myeloma
4. Myelosclerosis and fibrosis
5 Marble bone disease

E. Diseases involving the spleen
1 Idiopathic
2 Tuberculosis
3 Malaria
4 Kala Azar
5. Sarcoidosis
6. Collagen disorders
7 Obstruction of the splenic vein
8 Syphilis
9 Lymphoma
10 Felty's syndrome
11 Amyloidosis
12 Gaucher's disease
13 Liver disease

Enumeration of Reticulocytes

Technic

1. One drop of blood and two drops of a saline solution of brilliant cresyl blue are placed on a clean glass slide and mixed for

about 15 seconds with a tooth pick, wooden applicator, or the corner of a cover glass. A clean cover glass is then inverted onto the slide and gentle pressure is exerted on the cover glass with a piece of filter paper to make a thin preparation which is then sealed with vaseline.

2. After the preparation has stood for five to ten minutes, the proportion of reticulated red cells to total red cells is determined.

Comments

1. The above technic is the simplest method for enumerating reticulocytes and is as satisfactory as any other method which has been described.

2. As an alternative procedure an equal volume of blood and *a saline solution of brilliant cresyl blue may be allowed to stand* in a test tube for about 15 minutes. A wet preparation may then be made as described above or smears may be pulled in the usual fashion (p. 69), allowed to dry, and counterstained with Wright's stain.

3. It is essential that the smears be thin and the red cells evenly distributed. There should be no overlapping, clumping, rouleau formation, or crenation of the red cells.

4. Stain precipitated on red cells should not be confused with reticulocytes.

TABLE 20—*Variation in the Reticulocyte Count as Performed by Students*

Class	Mean	Range	2 S D	2 C V %
1	4 7	3 0 — 6 1	1 78	37.8
2	5 0	2 0 — 8.2	3 16	63.2
3	3.2	1.3 — 6 0	2.36	73.8

S D refers to standard deviation
C V refers to coefficient of variation

5. Crenation of the red cells may cause confusion, but the spine-like projections, although refractile, do not stain with brilliant cresyl blue.

6. When reticulocyte counts are made on the same preparation

by different observers the variations are great indeed. Repeated reticulocyte counts by the same observer on the same specimen of blood vary less. Therefore, it is recommended that one observer make all of the counts on any one patient.

7. The accuracy of students performing a reticulocyte count for the third time in their experience is illustrated in table 20. There were 50 students in each class. The "wet" method described above was used

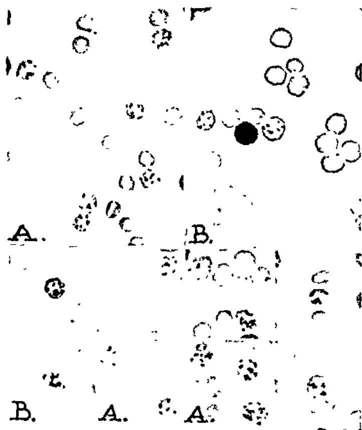


Fig. 4—Reticulocytes (x 900) A Stained with brilliant cresyl blue and counterstained with Wright's stain B Wet preparation, stained with brilliant cresyl blue.

Interpretation

1. In the wet preparations the reticulum of the red cells, the nuclei of the white cells, and the platelets are stained blue (fig. 4b). In the preparations counterstained with Wright's stain the reticulum appears blue and the cells otherwise stain as they usually do with Wright's stain (fig. 4a).

2. All red cells that contain reticulum are counted as reticulocytes. In "old" reticulocytes, only a few blue granules or scattered threads may be found. Such cells should be counted as reticulocytes.

3. The proportion of reticulated red cells to total red cells is determined by examining consecutive fields of the smear under oil immersion. The counting is greatly facilitated by placing into the ocular of the microscope a screen of stiff paper, in the center of which a hole has been cut. In this way the size of the field is reduced.

4. The greater the number of total cells examined, the higher the accuracy of the count. The percentage of reticulocytes in 500 to 1000 cells is usually determined.

Reagent

Brilliant Cresyl Blue (*Saline solution*)
Brilliant cresyl blue 1.0 Gm.
Sodium citrate 0.4 Gm
Saline (0.85 per cent) to make 100 ml

3. Draw the diluting fluid (Rees-Ecker) to the 101 mark.
4. Shake the pipet for 5 minutes.*
5. Expel and discard the first 4 drops from the pipet.
6. Load both counting chambers of the hemocytometer.
7. Allow the preparation to stand in a moist chamber for 15 minutes. An inverted Petri dish with a piece of wet filter paper in the top makes a satisfactory chamber.
8. Under high power with the light partially cut down, count the platelets in the finely ruled center area of each chamber.

To identify the platelets, use should be made of the fine adjustment of the microscope in order to obtain the critical focus that reveals the characteristic, highly refractile, silvery appearance of the platelets. They are lilac-colored, one-seventh to one-half the diameter of the red corpuscles, usually oval, rod or comma-shaped. They may be seen singly or in groups. It is important to distinguish platelets from globules of oil, irregularly shaped debris floating on the upper layers of the fluid, strings of cocci, and tiny oily particles adhering to the counting chamber.

Precautions and Sources of Error

1. Numerous methods have been devised for counting platelets. No method is entirely satisfactory including the one outlined above. This is to be expected because platelets agglutinate, fragment, and disintegrate readily. Furthermore, because of their small size, platelets are not easily differentiated from particles of stain, debris, and fat. The accuracy of students performing a platelet count for the third time in their experience is shown in table 21. There were 50 students in each class. The method described above was used. Even with greater experience, the error of the method is about ±40 per cent (2 C.V.). For this reason, *the value obtained always should be checked by making a survey of the blood smear to determine whether the number of platelets is increased, normal, or decreased by comparison with the normal blood smear*

2. Sampling errors, equipment errors, technical errors, and the "error of distribution of the cells in the chamber" which apply to the enumeration of erythrocytes also apply to platelet counting.

*A pipet rotor such as the Bryan-Garrey (A. S. Aloe Co., St. Louis, Mo.) may be used

TABLE 21.—*Variation in the Platelet Count as Performed by Students*

Class	Mean $\times 10^3$/mm.^{3}	Range $\times 10^3$/mm.^{3}	2 S D	2 C.V. %
1	210	83 — 585	165.2	69.0
2	275	116 — 420	149.6	59.8
3	96	34 — 184	20.4	21.2

S D refers to standard deviation.
C V refers to coefficient of variation

Capillary blood, if used, must be free-flowing, or if venous blood is used, care must be taken to insure direct puncture of the vein, since platelets agglutinate and disintegrate when mixed with tissue fluid. All of the precautions in pipeting and filling the chamber which were discussed in regard to the enumeration of erythrocytes must be observed in performing a platelet count.

3. Since platelets tend to agglutinate on unclean glass surfaces, all glassware must be scrupulously clean. It is advisable to rinse the cover glass and hemocytometer with alcohol just before use. Care must be taken to prevent dust from falling on the glass surfaces.

4. The diluting fluid must be fresh and should be centrifuged or filtered before use. The diluting fluid should be stored in glass-stoppered bottles in the refrigerator when not in use. Sometimes it will be found that solutions which have stood for a time cause hemolysis of red cells. Such solutions should be discarded. The hemolysis is usually produced by formic acid formed by the oxidation of formaldehyde.

5. If large aggregates of platelets are present in the chamber, the platelets cannot be enumerated, and the procedure must be repeated.

6. If after the above precautions have been taken, there are still many bodies present which are difficult to differentiate from platelets, a so-called "blank count" may be obtained on the diluting fluid and the result subtracted from the platelet count.

7. A more satisfactory but somewhat more cumbersome method of collecting blood is to place 1 ml. of diluting fluid in a 5 ml. syringe, attach a sterile, dry needle and aspirate 1 ml. of blood from the vein, without stasis, by drawing the plunger to the 2 ml.

mark. The needle is then withdrawn from the vein and the blood contained in it aspirated into the syringe. The needle is removed, the diluted blood in the syringe thoroughly mixed and expelled into a collecting bottle. It is important to use a syringe with a snugly fitting plunger and needle. A final dilution of blood of 1/200 is then made by drawing this mixture to the 1.0 mark in the R.B C. pipet and subsequently filling it to the 101 mark with the diluent.

Calculation

Each finely ruled center square is 1 x 1 x 0.1 mm.; consequently the number of platelets in a total volume of 0.2 mm.[3] is counted. To obtain the number in one mm[3] of blood, the number of platelets counted is multiplied by 5 × 200 (dilution factor), or by a factor of 1000 The count is reported to 3 significant figures.

Reagent

Rees-Ecker Diluting Fluid

Sodium citrate 3.8 Gm.
Brilliant cresyl blue 0.05 Gm.
✓Neutral formaldehyde (38 per cent U.S.P.) 0.22 ml
Distilled water to make 100 ml.

Filter and centrifuge (2500 r.p m. for 30 minutes).

Store stock solution in a refrigerator in a well stoppered bottle.

Filter an aliquot immediately before use.

For best results this solution should not be more than one week old.

Brecher-Cronkite Method

Technic

1 Insert a 20 gauge needle, with a syringe attached, into the vein After a suitable volume of blood has been drawn into the syringe for other tests (V P R C. etc.), remove the syringe, leaving the needle in place.

2. The phase condenser produces a hollow cone of light, and the vertex of the cone must be in the plane of the microscope slide. Therefore, the position of the condenser is critical. Preliminary adjustment of the condenser setting is readily accomplished by placing a piece of lens paper on the counting chamber. As the condenser is moved up and down, the area illuminated by the condenser changes from a ring to a small dot and again to a ring. The smallest dot that can be produced represents the optimal height of the condenser.

3 The phase effect is produced when the image of the annular diaphragm of the condenser and the ring-shaped phase plate of the objective lie over one another. To insure this, the condenser must be centered while viewing the images of the annular diaphragm and of the phase plate through a telescopic eyepiece supplied by the manufacturer. This is difficult for the uninitiated to carry out because of confusing diffraction patterns that appear in the telescopic eyepiece. The difficulty can be readily overcome if the following procedure is used. after proper focusing and centering of light source, an empty counting chamber is put on the stage and the condenser, with a 43x annulus in place, is adjusted as described above. Using an ordinary 43x objective, the mirror is adjusted to give optimal illumination and the microscope is focused on the lines of the chamber. One eyepiece is then replaced by the special telescopic eyepiece. When the eyepiece is focused, a white ring is seen that represents the image of the condenser annulus. The next step is to remove the 43x annulus from the condenser (or to turn the condenser to zero position, depending on the make of the equipment), and to change to the 43x phase objective. A black ring will now be seen, representing the phase plate. This black ring is approximately the size of and in the position of the white ring seen before Having visualized the images of the condenser annulus and the phase plate, it is now easy to superimpose them by centering the condenser with the 43x annulus again in place. Since the black ring is slightly larger, a fine black concentric ring (no crescent) remains at the inner aspect of the white ring when the condenser is properly centered. Moving the condenser down gives a red color to the white ring; moving it up produces a blue color. In optimal position the ring is white. Moving the mirror

makes the ring gray instead of white. The ring must be uniformly white.

Reagent

Diluting Fluid

Ammonium oxalate 1.0 Gm.
Distilled water to make 100 ml.

This solution should be stored in the refrigerator and filtered before use.

REFERENCES

1. Brecher, G. and Cronkite, E P Morphology and enumeration of blood platelets J Appl Physiol 3: 365, 1950
2. ——, Schneiderman, M, and Cronkite, E P The reproducibility and constancy of the platelet count. Am J Clin Path 23. 15, 1953

Table 22—*Classification of Thrombocytopenic Purpura**

A Primary (Idiopathic)

B Secondary

1 Chemical vegetable, animal and physical agents
  a Chemical
    1) Myelosuppressive agents—Nitrogen mustards, TEM, Myleran, urethane, anti metabolites, benzol
    2) Organic arsenicals, sulfonamides, Sedormid, quinidine, quinine, gold salts, oxytetracycline, streptomycin, paraaminosalicylic acid, phenylbutazone, antipyrine, tridione, meparfynol, thiourea, dinitrophenol, digitoxin, mercurials, iodide, bismuth ergot, organic hair dyes, estrogens, etc
  b. Vegetable foods, orris root
  c Animal snake venoms, pertussis vaccine
  d Physical ionizing radiation, extensive burns, heat stroke
2 Blood disorders
  a Leukemias: acute, late stages of chronic
  b Anemias, hypoplastic, myelophthisic, pernicious anemia
  c Splenic disorders (table 19)
  d Thrombotic thrombocytopenic purpura
3. Infections septicemia, subacute bacterial endocarditis, typhus, measles, vaccinia

*Modified from Wintrobe, *Clinical Hematology* 4th edition Philadelphia, Lea and Febiger, 1956

Enumeration of Leukocytes

Technic

1. Draw blood to the 0.5 mark in the white cell pipet with great accuracy
2. Wipe off blood adhering to the outside of the pipet.
3. Draw diluting fluid ("Turk's" solution) to point 11 with great accuracy.
4. Shake the pipet for 3 minutes.*
5. Expel and discard the first 4 drops from the pipet.
6. Load both counting chambers of the hemocytometer from the one pipet.
7. Under low power, count the number of the leukocytes in each of the 4 large corner squares in each chamber.

In each large square begin counting at the extreme upper left and work to the right, down and to the left, and so forth. Count those cells which touch dividing lines to the left and above and omit those touching dividing lines to the right and below

All the suggestions and precautions concerning pipeting, shaking, filling the chambers, and counting, which apply to the enumeration of erythrocytes, also apply to the enumeration of leukocytes and are not repeated here

Sources of Error

Essentially all of the sampling, equipment, technical and distribution errors, which apply to enumerating erythrocytes, are also germane to the enumeration of leukocytes However, the dilution used in leukocyte counting is not as great as in red cell counting and the clinical significance of the leukocyte count does not require the degree of accuracy which is essential for the enumeration of red cells Assuming no technical errors, when one pipet is used, 4 large squares in each of two chambers counted, and the total leukocyte count is 7000 mm.^{3}, the error is ±15 per cent (2 C.V.) and the true count lies between 5900 and 8100 mm.^{3} The higher the total leukocyte count, the greater the number of cells counted, and the error diminishes In practice, it is common to count the

*A pipet rotor such as the Bryan Garrey (A S Aloe Co., St. Louis, Mo) may be used

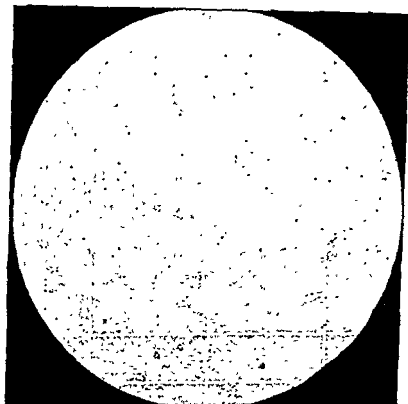


Fig 6—Leukocyte count The group of 16 squares shown is one of the corner squares of the nine large squares of the chamber The *accumulative count is as follows*

```
 3  →  4  →  6  →  9
                   ↓
21  ← 17  ← 14  ← 11
 ↓
23  → 24  → 24  → 26
                   ↓
37  ← 35  ← 30  ← 28
```

leukocytes in 4 large squares of only one chamber This procedure saves little time and is less desirable since the error is ±20 per cent (2 C.V).

The accuracy of students performing a leukocyte count for the fifth time in their experience is shown in table 23. There were 50 students in each class. The method recommended above was used.

TABLE 23—*Variation in the Leukocyte Count as Performed by Students*

Class	Mean $\times 10^3$/mm³	Range $\times 10^3$/mm³	2 S D	2 C V %
1	45 1	29 0 — 56 2	11 4	25 2
2	9 7	7.3 — 13 0	2 6	26 8
3	32 3	19.3 — 55 0	15 8	48 8

S D refers to standard deviation.
C V refers to coefficient of variation

Calculation

The volume of diluted blood contained in one large square (1 x 1 x 0 1 mm.) is 0.1 mm.³ The general formula for the calculation of the leukocyte count is

$$\frac{\text{No. of cells counted} \times \text{dilution} \times 10}{\text{No. of large squares counted}} = \text{No. of cells mm.}^3$$

The dilution in the white cell pipet is 1/20 when blood is drawn to the 0.5 mark. Therefore, when 8 large squares are counted, the sum of the numbers of cells counted is simply multiplied by 25 to give the number of leukocytes in 1 mm³ of blood.

If the total leukocyte count is below 2500/mm³, blood is drawn to the 1 0 mark instead of to the 0.5 mark and diluted to the 11 mark, thus producing a dilution of 1/10 instead of 1/20. The sum of the numbers of cells counted in 8 large squares is then multiplied by 12 5 instead of 25.

If the leukocyte count is markedly elevated, as in many patients with leukemia, a dilution of 1/100 or 1/200 is made in the red cell pipet The sum of cells counted in 8 large squares is then multiplied by 125 or 250, respectively

The dilution fluid used in counting leukocytes produces hemolysis of all of the nonnucleated erythrocytes. Nucleated red cells are not destroyed and will be included in the leukocyte count If their

number is great they should be subtracted from the leukocyte count. This can be done by determining the number of nucleated red blood cells (N.R.B.C.) per 100 leukocytes in the blood smear. Then,

$$\frac{\text{No. of N.R.B.C.}}{100 + \text{No. of N.R.B.C.}} \times \text{Leukocyte Count} = Y$$

$$\text{Leukocyte Count} - Y = \text{Adjusted Leukocyte Count}$$

For example, if the total "leukocyte" count is 7000 per mm.[3] *and* in the blood smear there are 10 nucleated red cells per 100 leukocytes, then the adjusted count is

$$\frac{10}{100 + 10} \times 7000 = 637$$

$$7000 - 637 = 6363 \text{/mm.}^3$$

Reagent

Turk's Diluting Fluid
Glacial acetic acid 3 ml
Gentian violet 1 ml.
Distilled water to make 100 ml.

Enumeration of the Absolute Number of Eosinophils

Technic

1. Draw blood to the 1.0 mark in the white cell pipet with great accuracy
2. Wipe off blood adhering to the outside of the pipet
3. Draw the eosinophil diluting fluid to point 11 with great accuracy
4. Repeat the above procedure with a second pipet.
5. Shake the pipets for 2 minutes

6. Expel and discard the first 4 drops from each pipet.
7. Immediately load one counting chamber with each pipet.
8. Allow 15 minutes for proper lysis and staining. The chamber should be covered during this period with an inverted Petri dish with a piece of wet filter paper in the top.
9. Enumerate all of the eosinophils in all 9 large squares in each of two chambers by the use of the low power magnification.

Comments

1. Either capillary or venous blood may be used. If the latter is used, the eosinophils should be enumerated within 4 hours after the blood has been drawn.
2. The eosinophils should be enumerated within 3 hours after the blood has been diluted. Allowing the eosinophils to stain for a longer period of time results in a decline in the number.
3. With the stain used, only the eosinophils should be visible. The granules stain red. The nuclei do not stain.
4. All of the suggestions and precautions concerning pipeting, shaking, filling the chambers, and counting which apply to the enumeration of erythrocytes also apply to the enumeration of eosinophils and are not repeated here.
5. Because of the small number of total cells counted, the error in the method is greater than the error involved in enumerating erythrocytes. When two pipets and two chambers (Neubauer ruling) are used, the inherent error in the count, expressed as 2 C.V. is approximately 35 per cent when the level of eosinophils in the blood is 175 mm.³ To partially overcome this large inherent error several special types of counting chambers have been devised to allow for a greater number of eosinophils to be counted. The Fuchs-Rosenthal ruling consists of sixteen 1 mm. squares bordered by triple lines, with each 1 mm. square subdivided into sixteen smaller squares by single lines. Hemocytometers with two Fuchs-Rosenthal rulings, each with a depth of 0.2 mm. rather than the standard 0.1 mm. are available. The total volume of each chamber is 3.2 mm.³ rather than 0.9 mm.³ as in the standard 0.1 mm. deep chamber with the improved Neubauer ruling. The Speirs-Levy eosinophil counting chamber consists of ten 1 mm. squares arranged in two horizontal

rows of 5 each, with each square bordered by triple lines and subdivided by single lines into sixteen small squares. The Speirs-Levy counting chamber is 0.2 mm. deep, as in the Fuchs-Rosenthal chamber, and four such chambers are provided on each hemocytometer. *Since the accuracy of the count is proportional to the square root of the number of cells counted, the Fuchs-Rosenthal or Speirs-Levy chamber is preferred to the standard Neubauer chamber.* For example, when 2 pipets are used and all of the eosinophils in the 4 Speirs-Levy chambers are counted, the inherent error in the count, expressed as 2 C.V. is approximately 18 per cent when the level of eosinophils in the blood is 175 mm.[3]

Calculation

1. When all of the eosinophils are counted in two Neubauer chambers and the initial dilution is 1 to 10,

$$\frac{\text{No. of eosinophils counted} \times 10}{1.8} = \text{Eosinophils mm}^3$$

2. When all of the eosinophils are counted in two Fuchs-Rosenthal chambers and the initial dilution is 1 to 10,

$$\frac{\text{No. of eosinophils counted} \times 10}{6.4} = \text{Eosinophils mm}^3$$

3. When all of the eosinophils are counted in four Speirs-Levy chambers and the initial dilution is 1 to 10,

$$\frac{\text{No. of eosinophils counted} \times 10}{8} = \text{Eosinophils mm}^3$$

Reagent

Eosinophil Diluting Fluid (Pilot's Solution)

Propylene glycol 50 ml.
Distilled water 40 ml
Phloxine (1 per cent aqueous stock solution) 10 ml.
Sodium carbonate (10 per cent aqueous stock solution) 1 ml.
Heparin sodium 100 units

Careful measurement of the sodium carbonate is necessary. Filter and keep in a well-stoppered bottle. This mixture remains stable and useable for at least one month at room temperature. The propylene glycol renders the erythrocytes invisible; the sodium carbonate lyses all leukocytes except the eosinophils; the phloxine stains eosinophil granules red; the heparin prevents clumping of the leukocytes.

REFERENCES

1 MacFarlane, J C W and Cecil, G W. Eosinophil counting. A modification of Pilot's method Brit Med J 2 1187, 1951
2 Best, W R and Sampter, M: Variation and error in eosinophil counts of blood and bone marrow Blood 6 61, 1951
3 Speirs, R S An improved eosinophil counting slide J Lab & Clin Med 39 963, 1952

The Blood Smear

Technic

Preparation of Glassware: A good grade of scrupulously clean glassware is essential. The glassware must be flat since blood will not spread on concave or convex surfaces The cover glasses should be 22 mm square and 0 13 to 0 17 mm. thick (no 1).

1. Gross cleaning, by either of the following methods is satisfactory (a) Wash with soap and water, then with abundant clean hot water. (never allow the water to cool before all soap has been removed), followed by distilled water Store in 95 per cent alcohol in a closed container (b) Soak in cleaning solution for 4 to 6 hours Then wash thoroughly in clean water, then distilled water Finally run through two thorough washings with 95 per cent alcohol. Store in alcohol in a closed container.

2 Fine cleaning is done by polishing the cover glass with a clean cloth (old linen preferable) absolutely free from dust and grease After the cover slips have been cleaned, they are handled by touching only the edges

Preparation of Smears (Cover-Glass Method) (figure 7):

1. Pick up a cover glass by two adjacent corners between the thumb and the index finger of the right hand. This leaves the left hand free to regulate the size of the drop to be picked up.

2. The cover glass is held just over a freshly drawn drop of capillary blood. Allow the blood to reach the cover glass by capillary attraction. Touching the skin with the cover glass must be carefully avoided. The drop should be about 2 to 3 mm. in diameter.

3. Pick up a second cover glass with the left hand and hold by two adjacent corners between the thumb and the index finger.

4. Gently place the cover glass held in the right hand over the cover glass in the left hand in such a manner that the two superimposed cover glasses form an octagon. If the glassware is clean, the blood will spread quickly and evenly between the two surfaces.

5. The protruding corner of the cover glass on top is grasped between the thumb and index finger of the right hand. A moment is

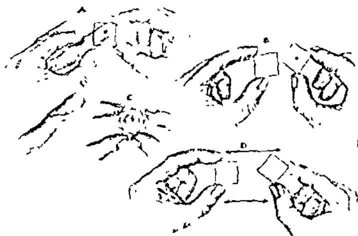


Fig. 7.—The preparation of blood smears by the cover-glass method

allowed for the spreading to occur and just before it is completed, the two cover glasses are separated by a rapid but steady, absolutely horizontal, lateral pull. If the cover glasses are not drawn in a strictly horizontal direction, an even distribution of cells will not

result. Care must also be taken to avoid a squeezing pressure between the two glasses. Excessive delay in separating the cover-glasses will result in clotting, and it will be found difficult to pull them apart. If sufficient time is not allowed for spreading, the smear will cover a small area and be too thick. If the operation has been carried out successfully, the spread will be smooth and even with the red blood cells lying flat rather than in rouleau, and the edges of the cells will be just touching but not overlapping. At least 8 such areas (low power) constitute the minimal requirements for a good smear.

6 Place the preparations (smear side up) on clean paper and allow them to dry in air

7 *Smears should always be made from capillary blood or from freshly drawn venous blood to which NO anticoagulant has been added* The morphology of the leukocytes is considerably distorted by anticoagulants and identification of these cells is made extremely difficult If venous blood is used, all but several drops of blood are discharged from the syringe into the bottle containing the anticoagulant With the syringe in a vertical position, pressure is applied on the barrel until a small drop of blood extrudes from the tip The cover glass is then held just over the tip. The smears should be stained within a few hours

Preparation of Wright's Stain: A solution of Wright's stain certified by the Commission on Staining may be purchased and is entirely satisfactory

Wright's stain (National Aniline) in powdered form, certified by the Commission on Staining, may also be purchased A solution is prepared by grinding 0.1 Gm. of powder with a few ml. of absolute methyl alcohol (C P , acetone-free and chemically pure) in a mortar Slowly add the alcohol, a few ml at a time, until 60 ml of alcohol have been added, then grind thoroughly This process should take from 20 to 30 minutes All the stain must go into solution If possible, allow to stand one to two days. Filter before use All the glassware used in the preparation and storage of the dye solution must be absolutely dry The reagent bottle must be well-stoppered to prevent evaporation and to prevent water vapor from being taken up by the alcohol

Wright's stain does not deteriorate on standing under the above conditions

Preparation of Wright's Stain Buffer (pH 6.4)

Primary (monobasic) potassium phosphate (KH_2PO_4), anhydrous 6.63 Gm.

Secondary (dibasic) sodium phosphate (Na_2HPO_4), anhydrous 2.56 Gm.

Distilled water to make 1 liter.

If a more alkaline buffer is desired (pH 6.7) it may be prepared as above but with 5.13 Gm. of anhydrous primary potassium phosphate and 4.12 Gm. of anhydrous secondary sodium phosphate.

Staining of Smears:

1. Place the air-dried blood film with the smeared side upwards on a support (an inverted cork fixed by means of paraffin to the bottom of a pan) from which it can readily be picked up.

2. Cover the smear completely with Wright's stain, and allow the stain to remain on the smear for 2 minutes. This procedure fixes the smear.

3 Add an equal volume of buffer and to obtain mixing blow gently on the surface to set up slow currents. Allow to stand for 3 minutes. A greenish metallic scum should appear and the margins should show a reddish tint. In adding the buffer, care should be taken that the solution does not run over the edges of the cover glass. It should be noted that each fresh batch of stain requires a new formula for stain and buffer combination. In general, equal numbers of drops are used. However, it may be necessary to alter the relative amount of stain and buffer and the time of staining in order to obtain satisfactory preparations.

4. Float off, rather than wash off, the scum with a stream of water (preferably distilled) aimed on an angle at one corner of the cover glass. *The stream is first run very slowly and then more* briskly, so as to free the smear from all traces of excess stain All this time the cover glass must remain absolutely horizontal Washing should take from 5 to 10 seconds. If a stream of water from an overhead water bottle is not available, a dropper may be used to float off the scum, the cover glass then placed on an angle, and the smear washed by forcing a jet of water against it

5. After washing is completed, the cover glass is tilted and the lower edge touched to a blotter so that the excess water is quickly drained off.

6. Dry the smear by placing against some support and letting one edge rest on a blotter, or dry by waving it gently in the air, touching only the edge of the cover glass.

7. Remove the stain on the back of the preparation by gentle rubbing with gauze moistened with alcohol

8. Mount the cover glass, stained side down, onto a slide by means of neutral Canada Balsam. Enough mounting fluid should be used to spread completely under the cover glass so as to avoid air pockets

Comments

1. *In a good stain*, the film will appear pink to the naked eye; microscopically the erythrocytes will be pink rather than lemon-yellow or red, the nuclei of the leukocytes purplish blue, neutrophilic granules violet-pink or lilac, eosinophilic granules red, and the basi and oxychromatin of the nuclei will be clearly differentiated The areas between the cells will be clear with no film or precipitated stain visible.

2. *If the smear is excessively blue*, this may be due to (a) too thick a smear, (b) insufficient washing, (c) too prolonged staining time, or (d) excessive alkalinity of the stain, buffer or water. In such preparations nuclear chromatin is deeply stained, and erythrocytes stain blue or green. The cytoplasm of the lymphocytes becomes gray or lavender and the granules of the neutrophils become intensely overstained and appear larger than normal. Eosinophil granules become deep gray or blue. To improve the color, less stain or more diluent may be used, or the time of staining (step 3) may be decreased and the time of washing increased. If the buffer is too alkaline and the above adjustments do not correct for this, the buffer should be remade.

3 *If the whole stain is too red*, this is due to excess acidity of the stain, buffer, or water In acid preparations, nuclear chromatin is stained pale blue rather than a vivid blue, the erythrocytes stain bright red or orange rather than pink, and eosinophil granules stain a brilliant red A common cause of excess acidity is exposure of the stain or buffer to acid fumes. This must be avoided To correct for excess acidity it is usually necessary to use a fresh batch of

stain or buffer or both. If the tap water is alkaline, substitution of tap water for distilled water in washing may be of some benefit.

4. If the nuclei, erythrocytes, and eosinophilic granules are pale, this is due to (a) excess washing or (b) understaining (step 3). To correct for this, decrease the time of washing or increase the time of staining.

5. *If there is scum or precipitated stain between the cells,* this is due to (a) unclean cover glass, (b) faulty washing because of failure to hold the preparation horizontal and to float off the scum, or (c) permitting dust to settle on the smear. This pitfall may be prevented by using clean cover glasses, by holding the preparation horizontally during the washing, by learning to play the stream of water to the best advantage in washing, and by keeping the smears clean during the interval which elapses between pulling and staining.

Examination of the Blood Smear

The preparation should be examined first under low-power to determine if the distribution of the cells and the staining characteristics are satisfactory. Even for the most experienced and talented observer, an unsatisfactory preparation has no value and, worse still, it may be seriously misleading. A well made, well stained blood smear is an absolutely essential prerequisite for the examination of erythrocytes, platelets and leukocytes. It is a complete waste of time to attempt to examine an unsatisfactory preparation.

If the preparation is found to be satisfactory, *the best area is selected for detailed examination* (fig. 8). This area is then placed under the oil-immersion objective. It is important to choose an area where the edges of the erythrocytes are nearly, but not quite, touching one another. If the area is too thick, the erythrocytes and leukocytes will appear small and the detailed morphology will be obscured. If the area is too thin, the erythrocytes will appear larger and better filled with hemoglobin than they actually are. The better the preparation, the easier, more rapid, and more reliable is the examination.

It is recommended that the student follow a set procedure in the examination of the smear The recommended routine is outlined in table 24 and briefly discussed below If the neophyte routinely

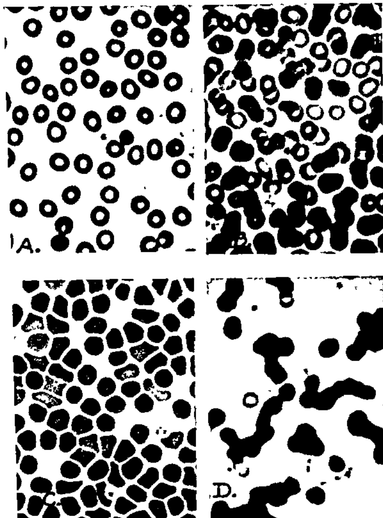


Fig 8—Photomicrographs of blood smears stained with Wright's stain (x 900) A, B and C are from the same smear A The red cells are spaced neither too thickly nor too thinly In an area such as this, the detailed morphology of the red cells may be examined B This area is too thick for a detailed examination of red cell morphology C Photomicrograph taken at the edge of the smear The red cell morphology is distorted Many of the cells are "squared" Some of the cells take on the appearance of spherocytes Examination of a blood smear in such an area as this may be grossly misleading D. Rouleau formation.

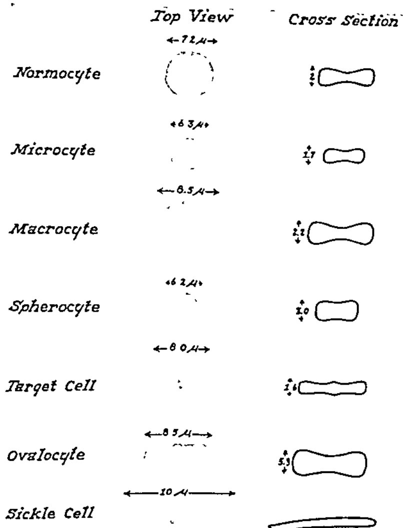


Fig 9—Variations in the size and shape of erythrocytes

follows this outline from the beginning, interrogating himself regarding each point, eventually the p[illegible] will become established and it is less likely [illegible] import[illegible] of the examination [illegible]

TABLE 21—*Outline for the Examination of a Blood Smear*

A *Erythrocytes*
1 Size
a Anisocytosis
b Normocytic
c Macrocytic
d Microcytic
2 Shape
a Poikilocytes
b Spherocytes
c Target Cells
d Ovalocytes
e Sickle Cells
3 Color
a. Normochromic
b. Hypochromic
4 Abnormal Forms
a Polychromatophilia
b. Stippling
c. Cabot Rings
d Howell-Jolly Bodies
e Nucleated RBC
f Parasitized RBC
g Rouleau Formation

B *Platelets*
1 Estimation of the number
2 Morphologic abnormalities

C *Leukocytes*
1 Estimation of the number
2 Differential leukocyte count
3 Morphologic abnormalities
a Multi-segmentation of the nucleus of neutrophils
b "Toxic" forms (such as "toxic" granulation of neutrophils)
c Nuclear degeneration
d Vacuolization and degeneration of cytoplasm of lymphocytes

A. The erythrocytes should be inspected in regard to (1) size, (2) shape, (3) color, and (4) abnormal forms (figures 9, 10 and 11).

1 *Size* With experience it can be determined with considerable reliability if the cells are on the average macrocytic, normocytic or microcytic The degree of variation in size (anisocytosis) should be estimated and recorded as 0 to 4 plus

2 *Shape* The presence of abnormally shaped cells such as spherocytes, target cells, ovalocytes and sickle cells must be carefully looked for since the presence of these cells may have considerable significance diagnostically. The degree of variation in shape (poikilocytosis) should be estimated and recorded as 1 to 4 plus

3 *Color* The intensity of the color of the erythrocytes can be

used to estimate the mean corpuscular hemoglobin concentration with a high degree of accuracy. *Hypochromia, if present, should* be graded 1 to 4 plus.

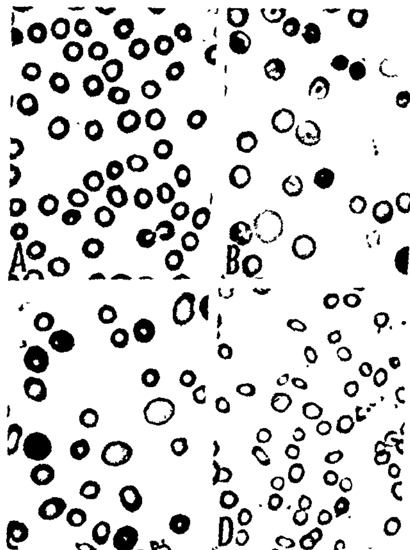


Fig 10—Photomicrographs of blood smears stained with Wright's stain (x 900) A Normal erythrocytes B Anisocytosis, many target cells nucleated red blood cell and a target cell with two Howell Jolly bodies C. Macrocytic anemia D Microcytic, hypochromic anemia

4. *Abnormal Forms.* Polychromatophilia, stippling, Cabot-rings, Howell-Jolly bodies, nucleated RBC, parasitized RBC, and rouleau

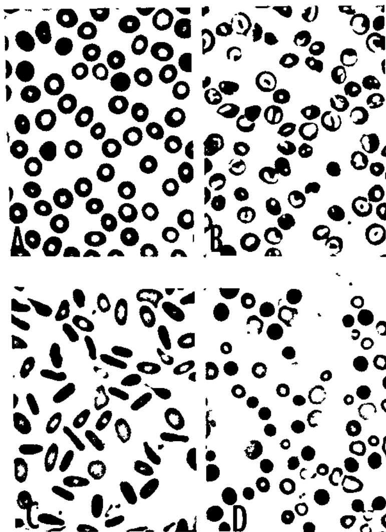


Fig 11 —Photomicrographs of blood smears stained with Wright's stain (x 900) A Normal erythrocyte morphology B Target cells (Hemoglobin C trait) C Hereditary elliptocytosis D Hereditary spherocytosis The small dense cells are spherocytes

formation should be noted. The degree of polychromatophilia can be estimated and reported as 1 to 4 plus. The number of nucleated RBC are expressed per 100 leukocytes observed in the differential count.

B. Platelets

1. The number of platelets is estimated. This is done by surveying several oil-immersion fields and estimating whether the number is normal, increased or decreased, as compared with the frequency with which they are observed in normal blood. In so doing, it is important to first make certain under low-power that the platelets are evenly distributed and not agglutinated in one area of the smear.

2. Variation in size and morphology of the platelets may be present in disease. Platelets normally appear granular and measure 2 to 5 μ in diameter. In disease, the size may vary from a fraction of a μ to that of a mass of agranular cytoplasm the size of a red cell (7μ).

C. *The leukocytes should be studied with regard to (1) number*, (2) type, and (3) morphologic abnormalities.

1. The leukocyte count can be estimated by surveying several oil-immersion fields and determining whether the number is normal, increased, or decreased, as compared with the frequency with which they are observed in normal blood.

2. Differential leukocyte counting is done under oil-immersion with a mechanical stage. Care should be taken to avoid counting the same area twice. This is done by moving from left to right, down one field, right to left, etc. One hundred leukocytes are usually counted. The actual number may be varied according to the number of leukocytes present. When there is leukopenia, fewer than 100 may be examined, whereas, when there is leukocytosis, more cells should be studied. In order to obtain the true proportion, it is necessary to include unclassified cells as well as those which can be classified. The greater the number of cells examined, the greater is the degree of accuracy. For example, if the true neutrophil count is 50 per cent and 100 cells are counted the error is ± 15 per cent. If 200 cells are counted the error is ± 11 per cent; if 400 cells are counted the error is ± 7 per cent In order to double the accuracy of a count it is necessary to count four times the number of cells, and to triple it, nine times. A leukocyte count should always be

made at the time a differential is done. It is only by this means that absolute rather than relative changes in the number of cells can be determined. The absolute number for a particular cell type is obtained by multiplying the total leukocyte count by the per cent obtained in the differential count.

In differentiating leukocytes it is important that all of the detailed characteristics recorded in tables 25 and 26 be observed. It should become a habit to note the following characteristics in examining a cell:

- (a) The over-all size of the cell
- (b) The nucleus
    - (1) Position
    - (2) Shape
    - (3) Color
    - (4) Chromatin structure
    - (5) Nuclear membrane
    - (6) Presence or absence of nucleoli
- (c) The cytoplasm
    - (1) Relative amount (nuclear-cytoplasm ratio)
    - (2) Color
    - (3) Presence or absence of perinuclear clear zone
    - (4) Granules-number, color, size and distribution.

The student should be careful to identify a cell by all of these characteristics and not be guided by one cell characteristic alone.

3. The presence of certain morphologic abnormalities such as multi-segmentation of the nucleus of neutrophils, giant metamyelocytes, deeply staining basophilic granules ("toxic granulation") in the neutrophils, nuclear degeneration, vacuolization and degeneration of cytoplasm may be of considerable prognostic and diagnostic significance.

Value of the Blood Smear

More valuable information may be obtained from the proper examination of the blood smear by a competent observer than can be obtained from any other single laboratory examination in medicine However, a knowledge of the limits of normal variation is

TABLE 25—*Morphologic Characteristics of the Myeloid Cells (Wright's Stain)*

Cell Type	Size μ	Nucleus			Cytoplasm		Granules		
		Nucleoli	Chromatin	Shape	Relative Amount	Color	Number	Size	Color
Myeloblast	10–18	2–5	Fine	Oval	Scanty	Deep blue	None		
Promyelocyte	12–20	2–4	Medium	Oval or round	Moderate	Light blue	Few	Medium large	Azure
Myelocyte	12–18	Absent	Medium	Round or oval	Moderate	Bluish pink	More	Medium	Blue-black

Metamyelocyte		10–18	Absent	Coarse	Band	Plentiful	Pink	More	Medium	Pink red or black
PMN Neutrophil		10–15	Absent	Coarse	2–5 lobes	Plentiful	Pink	Many	Small	Violet pink
PMN Eosinophil		10–15	Absent	Coarse	2–3 lobes	Plentiful	Pink	Many	Large	Red
PMN Basophil		10–15	Absent	Coarse	2–3 lobes	Plentiful	Pink	Many	Large	Black

TABLE 26 —*Morphologic Characteristics of the Non-Myeloid Cells (Wright's Stain)*

Cell Type	Size μ	Nucleus			Cytoplasm		Granules
		Nucleoli	Chromatin	Shape	Relative Amount	Color	
Lymphoblast	10–18	1–2	Medium	Oval	Scanty	Deep blue*	None
Lymphocyte	7–18	Absent	Coarse	Indented	Scanty or moderate	Variable*	None or few azure granules
Monocyte	12–20	Absent	Fine	Oval notched horseshoe	Abundant	Grayish-blue	Few lilac granules
Plasma cell	7–15	Absent	Very coarse	Round†	Abundant	Deep blue‡	None

*Perinuclear clear zone. †Eccentrically placed; ‡ Vacuoles in the periphery. Frequently there is a small relatively clear area in the cytoplasm

TABLE 27 — *Morphologic Characteristics of the Erythroid Cells (Wright's Stain)*

Cell Type	Size μ	Nucleus		Cytoplasm	
		Nucleoli	Chromatin	Color	Granules
Pronormoblast	14–19	Present	Fine	Blue	None
Basophilic normoblast	12–17	Absent	Less fine	Blue	None
Polychromatophilic normoblast	12–15	Absent	Medium	Bluish red	None
Orthochromic normoblast	8–12	Absent	Coarse	Red	None
eticulocyte	7–10	Reticulum		Red	None
hrocyte	7	None		Red	None

TABLE 28—*Relative and Absolute Normal Leukocyte Counts**
(per mm³)

Cell Type	Per Cent	Absolute Number		
		Mean	Minimum	Maximum
Total Leukocytes		7000	5000	10,000
Myelocytes	0	0	0	0
Metamyelocytes	3–5	300	150	400
PMN Neutrophils	54–62	4000	3000	5800
Eosinophils	1–3	200	50	400
Basophils	0–0.75	25	15	50
Lymphocytes	25–33	2100	1500	3000
Monocytes	3–7	375	285	500

*Modified from Wintrobe, *Clinical Hematology*, 4th edition, Philadelphia, Lea and Febiger, 1956

TABLE 29—*Differential Diagnosis of the Common Forms of Leukemia*

	Acute Leukemia	Chronic Myelocytic Leukemia	Chronic Lymphocytic Leukemia
Age, years	1–20	20–40	40–80
Splenomegaly	+	++++	++
Adenopathy	+	+	++++
Fever	++++	±	±
Bone pain	++++	±	+
WBC ($10^3/mm^3$)	1–200	100–800	30–100
Differential	Blasts	Myelocytes Metamyelocytes PMN	Adult lymphocytes
Anemia	++++	++	+
Platelets	Markedly decreased	High early, decreased late	Normal early, decreased late

necessary before this information can be obtained. This knowledge comes only through experience. *It is, therefore, extremely important that medical students and physicians personally examine the blood smear on every one of their patients.* If every student will routinely make this an integral part of the study of every patient, in time he

will become extremely proficient, and the rewards will be great. Fewer patients will have to be referred to specialists and the cost of laboratory examinations for the patients will be reduced. The common practice of leaving the examination of the blood smear to a technician results in the loss of much information which could be gained. The hurried technician usually performs only a differential count and either through lack of time, training or a total picture of the patient fails to examine in detail the morphology of the erythrocytes, platelets, and leukocytes. Furthermore, the physician fails to increase his experience and proficiency, in time what little ability he possesses is lost, and when the diagnosis is not obvious by other means, the patient must be treated (usually unsuccessfully) in ignorance of the correct diagnosis or referred to a specialist whose only talent above that of the referring physician may be an ability to examine the blood smear.

Special Stains for Blood

Siderocyte Stain

Technic

1. Prepare a blood smear and stain with Wright's stain in the usual fashion.
2. After the preparation is dry, immerse the cover glass in the Prussian blue reagent for 10 minutes.
3. Wash the cover glass under a stream of distilled water until a pink color appears.
4. Allow to dry and mount with balsam.

Comments

1. Siderocytes are erythrocytes containing granules which give a positive Prussian blue reaction for iron. The iron-containing granules of siderocytes appear as basophilic granules, "Pappen-

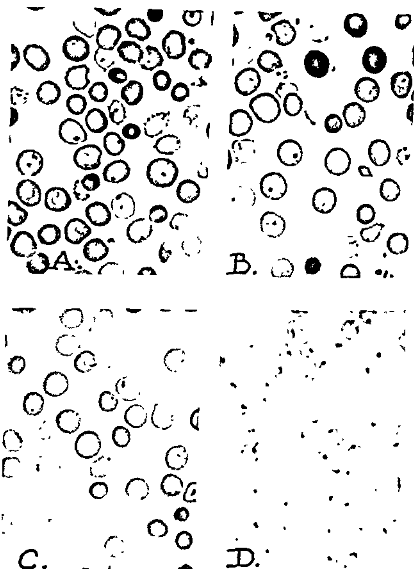


Fig 12.—Photomicrographs of blood smears (x 900) A Pappenheimer bodies (Wright's stain) B and C Siderocytes (Wright's stain counter stained with Prussian blue) D Heinz bodies (methyl violet stain)

heimer bodies," when blood films are stained with Wright's stain (fig 12 a).

2. The Prussian blue positive granules stain a vivid blue or blue-green. The staining characteristics of the cells themselves are the same as in the original films stained with Wright's stain although there may be some decolorization. The iron-containing granules vary in size from just within the range of visibility to large granules 2 microns in diameter. There are usually 1 or 2 granules per cell but there may be as many as a dozen. As a rule, the larger the granules, the less numerous they are (figs. 12b and c).

3. Siderocytes appear in the blood following splenectomy and may be seen in the blood of patients with unusual types of anemia. Siderotic granules are normally present in the normoblasts in the bone marrow and it has been suggested that these granules represent iron not immediately incorporated into heme. The granules are not found in the normoblasts of patients deficient in iron.

4. The technic described above can be applied without modification to smears of bone marrow and can be used with equal success on old smears stained with Wright's stain.

Reagents

Dilute hydrochloric acid solution
Concentrated hydrochloric acid 1 ml
Distilled water to make 100 ml

Potassium ferrocyanide solution (2 per cent)
Potassium ferrocyanide 2.0 Gm
Distilled water to make 100 ml

Prussian blue reagent
Dilute hydrochloric acid solution 75 ml
Potassium ferrocyanide solution (2 per cent) 25 ml.

The Prussian blue reagent must be made up immediately before use.

REFERENCES

1. Sundberg, R. D. and Broman, H. The application of the Prussian blue stain to previously stained films of blood and bone marrow. Blood 10: 160, 1955.
2. Douglas, A. S. and Dacie, J. V. The incidence and significance of iron-containing granules in human erythrocytes and their precursors. J. Clin. Path. 6: 307, 1953.

Heinz Body Stain

Technic

1. Place 2 drops of blood and 4 drops of a saline solution of methyl violet in a small test tube.
2. Mix gently and allow to stand at room temperature for 5 to 10 minutes.
3. Place a small drop of the mixture on a clean cover glass.
4. Invert the cover glass on a clean glass slide.
5. *Blot away the excess stain with a piece of filter paper and* rim the preparation with vaseline.

Comment

A semipermanent preparation may be made by placing a drop of the mixture on a cover glass. Prepare smears in the usual fashion: air-dry and then mount with balsam.

Interpretation

1. *The Heinz bodies appear as deep purple, irregularly shaped* bodies, varying in size from barely perceptible dots to spheres 2 microns in diameter (fig. 12d). One or more may be present within a cell. They are usually eccentrically placed, and lie close to the cell membrane. They may protrude from the erythrocyte by a stalk or occur free in the plasma.
2. Heinz bodies may be readily seen in a fresh, wet, unstained preparation of blood (p. 13). They appear as globular refractile bodies.
3. Heinz bodies stain with brilliant cresyl blue (reticulocyte stain) but less intensely than with methyl violet. They are not observed in the routine Wright's stain preparation because fixation with methyl alcohol removes the bodies.
4. Heinz bodies are frequently observed in patients with *hemolytic anemia, particularly in those caused by the ingestion of* toxic agents.

Reagent

Solution of methyl violet
Methyl violet 0.5 Gm.
Saline (0.85 per cent) to make 100 ml.

REFERENCE

FERTMAN, M H AND FERTMAN, M B: Toxic anemias and Heinz bodies. Medicine 34 131, 1955

Peroxidase Stain

Technic

1. Add 10 drops of solution A to a dry blood smear.
2. After 1.5 minutes add 5 drops of solution B. Allow it to stand for 3 to 4 minutes.
3. Wash thoroughly in tap water for 3 to 4 minutes
4. Counterstain with Wright's stain.

Interpretation

1. Peroxidase positive granules are found in myelocytes, metamyelocytes, neutrophils, and eosinophils (fig 13a) Myeloblasts, lymphoblasts, lymphocytes, and plasma cells are peroxidase negative (fig 13b).
2. The granules are small and blue-green in the myelocytes. In the neutrophils the granules are more abundant, large and blue-black The granules in the monocytes are like those in the myelocytes, but fewer and more scattered throughout the cell. Eosinophils have large bronze granules

Reagents

Solution A
Dissolve 0.3 Gm of benzidine base in 99 ml. of ethyl alcohol

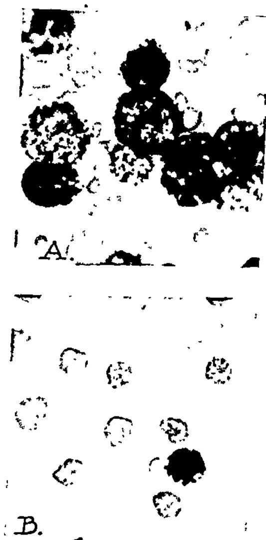


Fig 13.—Photomicrographs of blood smears Peroxidase stain (x 900) A Peroxidase positive myeloid cells from a patient with acute myeloblastic leukemia B Acute lymphoblastic leukemia The lymphoblasts are peroxidase negative There is a peroxidase positive neutrophil in the field

(95 per cent). To this add 1 ml. of a saturated aqueous solution of sodium nitroprusside This solution will remain active for 8 to 10 months.

Solution B

Add 0.3 ml of fresh 3 per cent hydrogen peroxide to 25 ml. of distilled water. This solution should be made just before use.

REFERENCE

Osgood, F. E. and Ashworth, C. M.: Atlas of Hematology. San Francisco. J. W. Stacey, 1937

Alkaline Phosphatase Stain

Technic

1. Prepare dry unstained blood smears in the usual manner.
2. Immerse the cover glass in the fixative solution for 30 seconds at 0 C. (±5°).
3. Wash in running water for 10 seconds.
4. Incubate in substrate mixture for 10 minutes at room temperature.
5. Wash in running water for 10 seconds.
6. Counterstain with Mayer's hematoxylin for 3 to 4 minutes.
7. Wash in running water for 10 seconds and air dry.
8. Mount with balsam and examine under high dry or oil immersion.

Interpretation

1. Neutrophils (metamyelocytes and polymorphonuclear) are the only cells which take up stain. The granules stain from a pale brown color to deep black. The amount of alkaline phosphatase positive material within a cell may be graded on the basis of intensity and appearance of the precipitated dye in the cytoplasm: 0, colorless; 1+, diffuse pale brown cytoplasm, no granules; 2+, brown cytoplasm with or without occasional clumps of brownish-black precipitate, 3+, brownish-black, unevenly distributed granular precipitate; 4+, uniform deep black granular precipitate.
2. In normal subjects, most of the neutrophils are unstained

TABLE 30.—*Alkaline Phosphatase Activity in Various Conditions*

Condition	Neg.	Per Cent of Neutrophils 1 Plus	2 Plus	3 Plus	4 Plus
Normal	64	30	6	0	0
Infection	6	24	10	20	40
Nonleukemic Myeloid Reactions	10	10	10	20	50
Acute Leukemia	100	0	0	0	0
Chronic Myelocytic Leukemia	99	1	0	0	0
Chronic Lymphocytic Leukemia	35	40	18	7	0

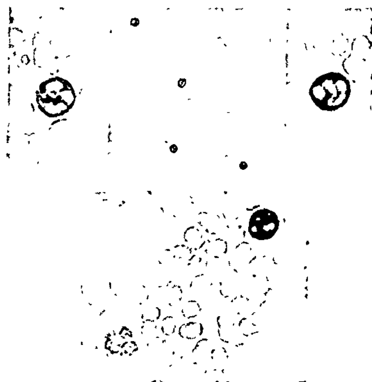


Fig 14.—Blood smears stained for alkaline phosphatase activity. Note the variation in staining intensity from cell to cell. Kindly furnished by Dr. L. S. Kaplow (Courtesy Blood)

(table 30). A few will be 1 to 2 plus. Alkaline phosphatase staining is increased in the neutrophils from patients with infection, polycythemia vera, myelofibrosis and other types of myeloid reactions. Alkaline phosphatase staining is decreased in the neutrophils from patients with acute leukemia and chronic myelocytic leukemia. The test is of value in distinguishing a nonleukemic myeloid reaction from chronic myelocytic leukemia.

Reagents

Fixative solution
Formalin (36 to 39 per cent formaldehyde) 10 ml.
Absolute methyl alcohol 90 ml.
Store in freezing unit of refrigerator.

Propanediol stock solution (0.2M)
2-amino-2-methyl-1, 3-propanediol 10 5 Gm.
Distilled water to make 500 ml.
Store this solution in the refrigerator.

Propanediol buffer (0.05M, pH 9.75)
Stock solution propanediol (0.2M) 25 ml.
Hydrochloric acid (O.1N) 5 ml.
Distilled water to make 100 ml
Store this solution in the refrigerator.

Substrate mixture (pH 9 5 - 9 6)
Sodium alpha napthyl acid phosphate 35 mg
Flast Blue RR 35 mg.
Propanediol buffer (0.05M) 35 ml
Filter and use at once.

Mayer's aqueous hematoxylin
Solution A
Hematein 1 0 Gm
Ethyl alcohol (90 per cent) 50 ml
Incubate 24 hours at 37 C

Solution B
Potassium aluminum sulfate 50 Gm.
Distilled water to make 1 liter

Mix A and B
Place in sunlight for 3 to 4 weeks.

2-Amino-2-methyl-1, 3-propanediol may be purchased from Eastman Kodak Co., Rochester, N. Y.; the sodium alpha napthyl acid phosphate from Dajac Laboratories, Leominster, Mass.; the Fast Blue RR (diazonium salt of 4-benzoyl-2: 5-methoxy-aniline) from General Dyestuff Corp., 255 Atlantic Ave., Boston, Mass.

REFERENCE

KAPLOW, L. S.: A histochemical procedure for localizing and evaluating leukocyte alkaline phosphatase activity in smears of blood and marrow. Blood 10: 1023, 1955.

The "L. E." Phenomenon

Clotted Blood Technic

Technic

1. Place about 10 ml. of freshly drawn blood in a clean, dry test tube.
2. Allow the blood to clot and stand in the test tube for about two hours at room temperature.
3. Decant the serum and discard.
4. Force the clot through a screen* of fine mesh to defibrinate it and collect the resulting fluid portion containing the cellular elements in a clean dry Petri dish or beaker.
5. Transfer the defibrinated blood to a hematocrit tube and centrifuge at 1000 R.P.M for 10 minutes
6. Withdraw and discard the serum down to the buffy coat.
7. Place a small drop of the buffy coat on a cover glass, make a thin smear in the usual fashion, and stain with Wright's stain

*Stainless steel wire gauze, 10 meshes to the inch. A small tea strainer which can be purchased at any hardware or grocery store is quite satisfactory

Rotary Method

Technic

1. Place 3 ml. of freshly drawn blood in a dry test tube (13 x 100 mm.) containing 1 mg. of sodium heparin (0 1 ml. of a solution containing 10 mg /ml.).
2. Incubate at room temperature for 1 to 2 hours.
3. Add 5 glass beads, 5 mm. in diameter, to the tube.
4. Rotate* the tube for 30 minutes at room temperature.
5. Fill a hematocrit tube with the blood and centrifuge at 1000 R.P.M for 10 minutes.
6. Withdraw and discard the plasma down to the buffy coat.
7. Place a small drop of the buffy coat on a cover glass, make a thin smear in the usual fashion, and stain with Wright's stain.

Comments

1. Production of the L.E phenomenon depends upon an immunocellular reaction which requires that at least three factors be brought together in vitro and that sufficient time elapse for the reaction The three known components are. (a) the L E factor which resides in the gamma globulin fraction of the serum and which is a nucleolytic agent, (b) cell nuclei, usually from PMN neutrophils or lymphocytes, with which the L.E. factor reacts; and (c) phagocytic cells, usually neutrophils, which engulf the lysed nuclear material

2 The typical L E cell is a mature neutrophilic or eosinophilic leukocyte which has engulfed a large purplish hyaline homogeneous mass The nucleus of the neutrophil is compressed around the periphery of the inclusion body The body, round or oval in outline, may vary in size from the size of a red cell to 3 or 4 times that size It may vary in appearance from granular to smoky to homogeneous No chromatin network is visible in the mass (fig. 15) These bodies stain less prominently than the host nucleus and take

*A satisfactory shaking device is the Bryan Garrey Blood Cell Pipette Rotor (A S Aloe Company) A platform style Kahn shaker can be used Petri dishes containing 3 ml of heparinized blood and 15 beads are agitated for 30 minutes in this type of shaker



Fig 15.—Blood smears stained with Wright's stain (x 900) Cells A through D are typical L.E. cells with a homogeneous inclusion body Cells E, F and G illustrate nucleophagocytosis Cell H, erythrophagocytosis. I, rosette with an inclusion body in the center J, two typical L.E. cells

a purplish stain, although there is variation in the staining from purple to reddish brown. In addition to the L.E. cells, the L.E. phenomenon includes the formation of clumps or rosettes. The rosette consists of a central homogeneous mass surrounded by neutrophilic leukocytes. Rosettes are easily visualized under the low power magnification of the microscope.

3. Nucleophagocytosis ("tart cells") must be differentiated from L.E. cells (fig 15). Nucleophagocytosis is a normal phenomenon and is present not uncommonly in preparations made to determine the presence or absence of L.E. cells. The phagocytized nucleus seen

in nucleophagocytosis can usually be differentiated from the L E. cell. The phagocytized nucleus has an intact, or nearly intact, nuclear pattern. It is frequently vacuolated, the chromatin is conspicuous and dense, and the chromatin around the circumference of the phagocytized nucleus is frequently condensed giving the impression of a thick rim. Eosinophilic staining of the nucleus may be prominent.

4. L E. cells are larger than other leukocytes and because of their size and distinctive appearance are readily observed under the low power objective of the microscope (fig 16). The entire area of at least two cover glasses should be systematically examined for at least 30 minutes before the smears are considered negative.

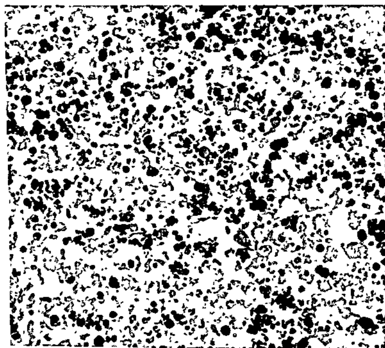


Fig 16 —Photomicrograph of a smear of the buffy coat of heparinized blood which had been rotated Wright's stain (x 200) Many L E cells are apparent The photomicrograph was kindly furnished by Dr W H Zinkham (Courtesy Bull Johns Hopkins Hosp)

The presence of L.E. cells should always be confirmed under the oil-immersion lens. A positive report should not be based on the finding of a single cell. The preparation should not be reported as positive unless at least several, *absolutely typical* L.E. cells are seen. In positive preparations many L.E. cells and rosettes are generally present.

5. The test may be falsely negative when severe leukopenia is present. This is because the reaction requires phagocytic cells in addition to the plasma factor. Therefore, if the patient has leukopenia, it is recommended that 5 ml. of the patient's serum be incubated with 5 ml. of washed cells obtained from a normal subject.

6. A negative test does not rule out the diagnosis of disseminated lupus erythematosus since the blood from some patients with obvious clinical disease cannot be demonstrated to form L.E. cells. An unequivocally positive test is probably diagnostic of the disease False positive tests rarely, if ever, occur. However, nucleophagocytosis may be mistaken for L.E. cells by the inexperienced.

REFERENCES

1. HARGRAVES, M. M. The L. E. cell phenomenon. *In* W. DOCK AND I. SNAPPER, Eds.: Advances in Internal Medicine. Chicago, The Year Book Publishers, Inc., 1954, vol. 6
2. CONLEY, C. L. The L. E. cell test. J. Chronic Dis. 5: 275, 1957

Examinations Concerned with Hemorrhagic Disorders

Cessation of loss of blood from a damaged vessel (hemostasis) is brought about not only as a consequence of the coagulation of blood and the formation of a firm fibrin clot (intravascular factors) but also by (1) prompt vasoconstriction and retraction of the vessel (vascular factors), and (2) by extravascular factors such as tissue tone, mass, tautness and resiliency. Thus, the causes of hemorrhage may be divided into three large groups (table 31)

TABLE 31—*Classification of the Causes of Hemorrhage*

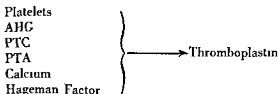
I Vascular Abnormalities
- A. Traumatic injury of vessels
- B Hereditary hemorrhagic telangiectasia
- C. Nonthrombocytopenic purpuras
  1. Allergenic purpura (anaphylactoid purpura, Henoch Schönlein purpura)
  2. von Willebrand's disease
  3. Hereditary familial purpura simplex
  4. Symptomatic infections, chemicals, animal agents, scurvy, certain chronic diseases
  5. Miscellaneous mechanical purpura. orthostatic purpura, purpura fulminans

II. Extravascular Abnormalities
- A. Atrophy of subcutaneous tissues (purpura senilis, purpura cachectica)
- B Fragility and hyperlaxity of the skin (Cushing's syndrome, Ehler Danlos syndrome, epidermolysis bullosa)

III Intravascular Abnormalities—Defects in Coagulation (see table 32)

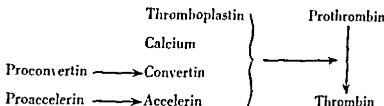
Coagulation of blood is initiated by contact of the blood with a "rough" surface and thus a series of reactions is started, which at least for the sake of simplicity and clarity may be divided into three stages.

In stage I, thromboplastin is formed. There are now known to be at least six factors concerned in the generation of thromboplastin. These are (1) platelets, (2) antihemophilic globulin (AHG), (3) plasma thromboplastin component (PTC), (4) plasma thromboplastin antecedent (PTA), (5) calcium, and (6) Hageman factor Thus, the first stage of coagulation may be diagrammed as follows



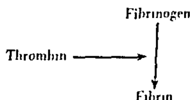
In stage II, prothrombin is converted into thrombin. This is brought about by thromboplastin and two co-factors, proconvertin

and proaccelerin. Calcium is also required. This stage may be represented diagrammatically as follows:



How these three factors and calcium interact to bring about the conversion of prothrombin to thrombin is not understood. It has been suggested that thromboplastin and convertin combine in the presence of calcium to bring about a minimal conversion of prothrombin to thrombin. The small amount of thrombin formed activates proaccelerin (proaccelerin→accelerin) and convertin and accelerin interact in the presence of calcium to form an intermediate compound, "prothrombinase," which then markedly accelerates the conversion of prothrombin to thrombin. Thus, there is an autocatalytic reaction which, after some thrombin has been formed, proceeds with ever increasing velocity until thrombin evolution is explosive.

In the third and last stage of coagulation thrombin converts fibrinogen into fibrin and a clot is formed. Thus,



This brief summary of the physiology of coagulation is unquestionably a naive oversimplification of the process. However, until a better understanding of the interaction of the individual components than now available is presented, it should serve as a framework of knowledge on which the student may add new contributions, as these are made available

The disorders of coagulation are classified in table 32 according to the stage at which the coagulation defect occurs. The prothrombin

consumption test is valuable in assessing the functional adequacy of stage I (thromboplastin formation). The prothrombin time is valuable in detecting an abnormality in stage II (thrombin formation). An abnormality in fibrin formation (stage III) may be suspected in the absence of a clot, in blood or plasma, with any of the tests.

TABLE 32—*Classification of the Hemorrhagic Disorders Due to Defects in Coagulation*

I Defect in Stage I—deficient thromboplastin formation
- A. Deficiency of AHG (Classical hemophilia, hemophilia A)
- B Deficiency of PTC (Christmas disease, hemophilia B)
- C Deficiency of PTA (Hemophilia C)
- D Thrombocytopenias (p. 62)
- E. Thrombocytopathic purpuras (thrombasthenia, Glanzmann's thrombasthenia) platelets numerically normal but morphologically and functionally abnormal
- F. Vascular hemophilia (AHG deficiency plus an abnormality of the capillaries)
- G Circulating antithromboplastin
  - 1 Idiopathic
  - 2. Associated with certain diseases, disseminated lupus, hyperglobulinemia, multiple myeloma, shock, hemophilia, pregnancy

II Defect in Stage II—deficient thrombin formation
- A Deficiency of prothrombin (Hypoprothrombinemia)
  - 1 Hereditary (rare)
  - 2 Acquired
    - a Idiopathic
    - b Liver disease
    - c Newborn
    - d Vitamin K deficiency—dietary or defective absorption
    - e Drugs—dicumarol and related compounds
- B Deficiency of Proaccelerin
  - 1 Hereditary (parahemophilia)
  - 2 Acquired
    - a Liver disease
    - b Newborn
    - c Leukemia
    - d Pernicious anemia
    - e Purpura fulminans
- C Deficiency of Proconvertin
  - 1 Hereditary
  - 2 Acquired

a. Liver disease
b. Newborn
c. Vitamin K deficiency
d. Drugs—dicumarol, Tromexan

III. Defect in Stage III—defective fibrin formation
A. Hereditary hypo- or afibrinogenemia
B. Acquired hypofibrinogenemia
1. Liver disease
2. Complication of pregnancy
3. Certain malignancies (prostate, stomach)
4. Certain bone marrow diseases
5. Pulmonary manipulation
6. Excessive fibrinolytic activity

In the initial laboratory study of a patient with a hemorrhagic disorder the following determinations should be performed: capillary fragility, bleeding time, whole blood coagulation time, clot retraction and enumeration of platelets (p. 51).

If there is thrombocytopenia, a positive capillary fragility test, a prolonged bleeding time, and poor and delayed clot retraction, then the etiology of the thrombocytopenic purpura must be determined.

If there is a defect in coagulation, which is not due to thrombocytopenia, then the prothrombin time (Quick) and prothrombin consumption tests should be performed.

If the defect is in the first stage of coagulation (poor prothrombin consumption) then the presumptive tests for the differentiation of *the types of hemophilia should be done*

If the defect resides in the second stage of coagulation (prolonged prothrombin time) then the presumptive tests for proaccelerin and proconvertin deficiencies should be performed.

The simple tests which are outlined herein are all that are usually needed for the diagnosis of the specific disorder of coagulation. However, it should be emphasized that in a few patients with a disorder of coagulation of mild degree, all of these tests may be normal and more elaborate tests may be necessary in order to establish the diagnosis. *If all of the tests are normal, a disorder of coagulation cannot be ruled out.*

The causes of alterations in the various tests are summarized

in table 33. The results of the tests in various disorders of coagulation are summarized in table 34. Synonyms for the coagulation factors are given in table 35.

TABLE 33.—*Causes of Alterations in the Various Tests of Coagulation*

I Positive tourniquet test
  - A Thrombocytopenic purpura—platelets too few to support capillaries under pressure
  - B Nonthrombocytopenic purpuras—damage to capillary endothelium
  - C Scurvy—deficiency of intercellular cement substance

II Prolonged bleeding time
  - A Thrombocytopenic purpura
  - B von Willebrand's disease—abnormality of capillaries
  - C Any of the causes under III and V—due to extreme poverty of coagulation factors

III Prolonged coagulation time
  - A Deficiency of AHG
  - B Deficiency of PTC
  - C Deficiency of PTA
  - D Deficiency of Hageman factor
  - E Deficiency of fibrinogen
  - F Deficiency of prothrombin and/or proaccelerin and proconvertin if deficiency is severe
  - G Idiopathic—unknown anticoagulants

IV Poor clot retraction
  - A Thrombocytopenia from any cause
  - B Thrombasthenia—platelets numerically adequate but functionally inadequate

V Prolonged prothrombin time
  - A Deficiency of prothrombin
  - B Deficiency of proaccelerin
  - C Deficiency of proconvertin
  - D Combinations of A, B and C

VI Reduced prothrombin consumption
  - A Deficiency of AHG
  - B Deficiency of PTC
  - C Deficiency of PTA
  - D Thrombocytopenia
  - E Thrombasthenia
  - F Circulating Antithromboplastin

TABLE 34.—*Coagulation Tests in Various Hemorrhagic Disorders*

Disease or Condition	Platelets Decreased	Capillary Fragility Increased	Bleeding Time Prolonged	Clotting Time Prolonged	Clot Retraction Poor	Prothrombin Time Prolonged	Prothrombin Consumption Reduced
Thrombocytopenic purpura	+	+	+	−[1]	+	−	+
Nonthrombocytopenic purpura	−	±	−	−	−	−	−
von Willebrand's disease	−	±	+	−	−	−	−
Thrombocytopathic purpura		±	−	−	+	−	+
AHG, PTC or PTA deficiency	−		[1]	+[2]	−	−	+
Hageman factor deficiency		±		+	−	−	+
Vascular hemophilia		±	+	+	−	−	+
Hypoprothrombinemia			−[1]	−[1]	−	+	−
Hypoproacclerinemia			[1]	−[1]	−	+	−
Hypoproconvertinemia			−[1]	−[1]	−	+	−
Hypofibrinogenemia		[1]	−[1]	+	−	+	−
Hyperheparinemia		−	−[1]	+	−	+[2]	+
Dicumarol excess		[1]	[1]	−[1]	−	+	−

[1]May be prolonged if condition is severe

[2]May be normal if condition is mild

TABLE 35.—*Synonyms for the Coagulation Factors*

Proaccelerin → Accelerin
Factor V → Factor VI
Labile factor
Plasma Ac globulin → serum Ac globulin
Plasma prothrombin conversion factor → serum accelerator
Proconvertin → Convertin
Factor VII
Stable factor
Plasma precursor → serum prothrombin conversion accelerator (Spca)
Antihemophilic Globulin (AHG)
Factor VIII
Antihemophilic factor (AHF) — A
Plasma thromboplastin factor (PTF) — A
Prothrombokinase
Thromboplastinogen
Plasma Thromboplastin Component (PTC)
Factor IX
Christmas factor
Antihemophilic factor (AHF) — B
Plasma thromboplastin factor (PTF) — B
Plasma Thromboplastin Antecedent (PTA)
Antihemophilic factor (AHF) — C
Plasma thromboplastin factor — C

Capillary Fragility

Technic

1 Apply a blood pressure cuff about the upper arm in the usual manner and take the blood pressure

2 Inflate the cuff to a point halfway between the systolic and diastolic pressures and maintain this level for 5 minutes.

3. Remove the cuff and examine the forearm, hands, and fingers for petechiae

4 The results of the test are graded in a rough manner from normal to 4+ as follows· 1+, few petechiae over the anterior surface of the forearm; 2+, many petechiae over the anteri:

surface of the forearm; 3+, multiple petechiae over the whole arm and dorsum of the hand; 4+, confluent petechiae in all areas of the arm and the dorsum of the hand.

Comments

1. This test is a crude measure of capillary fragility. The number and size of the petechiae are roughly proportional to the bleeding tendency. There is a fair degree of correlation between the number and size of the petechiae and the degree of thrombocytopenia. *However, the test also depends on the fragility of the capillaries and* may be markedly positive when the platelet count is normal. The results of the test are also influenced by the blood pressure, the thickness and texture of the skin, and the temperature of the skin.

2. The test should not be repeated on the same arm until one week later.

3. To make the tourniquet test more quantitative, a circle 5 cm. in diameter may be outlined in ink on the forearm, the upper edge of which is 4 cm. below the bend of the elbow anteriorly. Any petechiae present in the circle are noted and the test performed as above. The number of new petechiae in the area is noted. Normally there should be no more than 10 or at the most 20. The objection to this procedure is that the test may be markedly positive, yet only *a few of the petechiae may develop within the circle.*

4. Normally, petechiae frequently appear just below the lower level of the blood pressure cuff. Petechiae appearing in this location are discounted.

5. The results of this test on 100 medical students were as follows: negative, 72 per cent, 1+, 21 per cent, 2+, 7 per cent; 3 and 4+, zero per cent.

Bleeding Time

Technic

1. Place a blood pressure cuff on the arm above the elbow and inflate to 40 mm. Hg

2. With alcohol clean an area free of visible veins on the forearm.

3. Puncture the skin by means of a sterile spring lancet or a Bard-Parker blade to a depth of 2 to 3 mm. and a width of approximately 2 mm. A needle should *never* be used to make the puncture.

4. With a round piece of filter paper blot off the blood every 30 seconds. The drop of blood should never be wiped off. Only the tip of the drop should touch the filter paper. The blood should be allowed to flow onto the filter paper by capillary attraction. No manipulation of the wound is permitted.

5. Divide the number of drops of blood on the filter paper by 2 and the resulting figure represents the bleeding time in minutes.

Comments

1. The difficulty in producing a standardized wound limits the accuracy of the technic. It should be emphasized that the blade must be sharp and a visible cut must be produced.

2. A single normal bleeding time determination does not exclude the tendency to bleed; the test should be repeated in another area of the body if suspicion of hemorrhagic disease exists. Likewise, a single prolonged bleeding time does not prove the existence of a hemorrhagic disease. A large vessel may have been severed. The test should be repeated in another location.

3. The determination should be discontinued if the wound is still bleeding at 15 minutes and it should be reported as 15 minutes plus.

4. In general, there is a correlation between the bleeding time and the platelet count, the majority of patients with severe thrombocytopenia (less than 100,000/mm.^{3}) will have a prolonged bleeding time. The bleeding time of patients with a platelet count over 100,000/mm.^{3} will usually be normal. The rare capillary abnormalities in which the platelets are present in normal numbers are an exception to this rule.

5. The normal bleeding time as determined by 100 medical students using this method is 1 to 11 minutes (fig. 17). The median value is 2 minutes and the mean value is 2.9 minutes. In agreement with the observations of others, 87 per cent of the values fall between 1 and 4 minutes and there is a very long tail to the right where the curve appears to become asymptotic.

surface of the forearm; 3+, multiple petechiae over the whole arm and dorsum of the hand; 4+, confluent petechiae in all areas of the arm and the dorsum of the hand.

Comments

1. This test is a crude measure of capillary fragility. The number and size of the petechiae are roughly proportional to the bleeding tendency. There is a fair degree of correlation between the number and size of the petechiae and the degree of thrombocytopenia. However, the test also depends on the fragility of the capillaries and may be markedly positive when the platelet count is normal. The results of the test are also influenced by the blood pressure, the thickness and texture of the skin, and the temperature of the skin

2. The test should not be repeated on the same arm until one week later.

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5. The results of this test on 100 medical students were as follows: negative, 72 per cent; 1+, 21 per cent; 2+, 7 per cent; 3 and 4+, zero per cent

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Technic

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2. With alcohol clean an area free of visible veins on the forearm.

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4. With a round piece of filter paper blot off the blood every 30 seconds. The drop of blood should never be wiped off. Only the tip of the drop should touch the filter paper. The blood should be allowed to flow onto the filter paper by capillary attraction. No manipulation of the wound is permitted.

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5. The normal bleeding time as determined by 100 medical students using this method is 1 to 11 minutes (fig 17). The median value is 2 minutes and the mean value is 2.9 minutes. In agreement with the observations of others, 87 per cent of the values fall between 1 and 4 minutes and there is a very long tail to the right where the curve appears to become asymptotic.

REFERENCES

1. IVY, A. C., SHAPIRO, P. F., AND MELNICK, P.: The bleeding tendency in jaundice Surg Gynec. & Obst. 60. 781. 1935.
2 TOCANTINS, L. M: The bleeding time Am J. Clin Path 6: 160, 1936
3. O'BRIEN, J. R: The bleeding time in normal and abnormal subjects J Clin Path. 4: 272, 1951

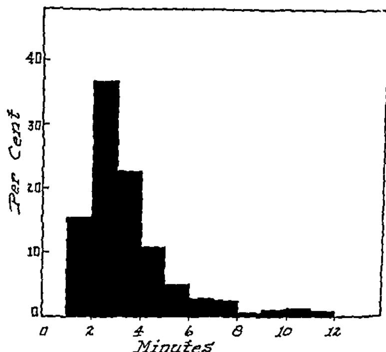


Fig 17—Frequency distribution diagram for the bleeding time performed on 100 presumably healthy, male medical students

Coagulation Time

Technic

1. With a tourniquet in place, 5 ml. of venous blood are drawn into a clean dry syringe.

2. One ml. of blood is placed into each of two, clean, dry, pyrex test tubes (13 × 100 mm.) with an inside diameter of 11 mm. The last three ml. of blood are discarded.

3. Gently tilt (about 45 degrees) the first tube at half minute intervals until no flow of blood is observed on complete inversion of the tube.

4. Then gently tilt the second tube at half-minute intervals until no flow of blood is observed on tilting.

5. The coagulation time is recorded as the time interval between the appearance of blood in the syringe and the time at which blood no longer flows on complete inversion of the second tube.

Comments

1. The coagulation time of blood is purely relative. The coagulation time of normal blood is influenced by temperature, the size of the tube, the volume of blood in the tube, the nature of the surface of the tube, the pH of the blood, the frequency and type of tilting movement, as well as other factors. A large number of modifications of the Lee-White method have been employed These vary as to the selection of the beginning time and the end point, the number and size of the tubes used, the temperature at which the determination is performed, the volume of blood in the tube, etc. No single technic has become standard. Therefore, it is important to perform the test under the same conditions each time and to always run a normal control. The normal mean value by the method described above is 9 minutes, and 95 per cent of the determinations fall between 3 and 15 minutes The distribution of the values as performed by, and on, 261 male medical students is shown in figure 18.

2 The site of the venipuncture should not be traumatized or squeezed.

3. It is important that the vein be entered quickly and neatly at the first attempt, as otherwise tissue juice will enter the syringe and shorten the coagulation time. If the vein is not entered on the first attempt, the needle and syringe should be discarded and another sterile syringe and needle employed.

4 The syringe should be clean and all possible air should be expelled from the syringe. The needle must fit tightly. Care should

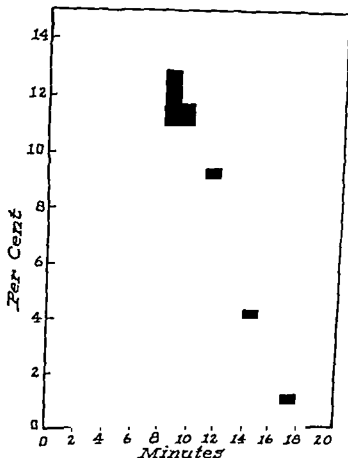


Fig 18.—Frequency distribution diagram for the coagulation time performed on 261 presumably healthy, male medical students

be exercised not to use too vigorous suction in filling the syringe, so that no air bubbles pass through the blood. Air tends to hasten coagulation.

5. The tubes must be scrupulously clean and smooth. Unclean or rough glassware hastens coagulation.

6. The inside diameter of the tubes should be constant. Blood clots more rapidly in smaller tubes. It is recommended that tubes with an inside diameter of 11 mm. be used

7. The needle should be removed from the syringe and the

blood allowed to run down the side of the tube gently to avoid agitation. Excessive agitation tends to hasten coagulation.

8. Temperature markedly influences the speed of coagulation Temperatures below 37 C. retard coagulation. Body temperature (37 C.) is the most desirable temperature at which to perform the test since it is at this temperature that coagulation takes place physiologically. It is therefore recommended that if possible the test always be carried out by placing the tubes in a water bath at 37 C.

9. The end-point is purely relative and does not indicate that complete coagulation has occurred in the whole column of blood. It is simply that time when sufficient fibrin has formed at the air interface to support the column of blood when the tube is completely inverted.

10 It must be emphasized that this test is only a rough screening procedure. Slight but significant prolongations of the clotting time cannot be detected in glass tubes and the test does not detect hypercoagulability.

REFERENCES

1 Lee, R I and White, P D · A clinical study of the coagulation time of blood Am J Med Sci 145· 495, 1913

2 Tocantins L M The Coagulation of Blood, Methods of Study New York, Grune & Stratton, 1955

Clot Retraction

Technic

1 The clot in one of the tubes used in the determination of the clotting time is separated from the wall of the tube by gently rimming the top of [illegible]

2 The tube
water bath at 37
of retraction, namely, separation of clot with expression of serum. The degree (*slight, moderate, marked*) as well as the rate of clot retraction should be observed

Comments

1. Mild shaking or jarring of the tube should be avoided since blood with delayed or no retraction may retract when subjected to shaking.

2. Normally, retraction of the clot from the walls of the test tube will have commenced by 30 minutes, is appreciable in 1 hour, and is complete by 24 hours. In abnormal conditions, clot retraction may not occur at all in 24 hours, or there may be variable degrees of retraction.

3. The retractility of the clot depends primarily on the number of platelets present. If the platelet count is less than 100,000 per $mm.^3$, poor retractility is usually present. Conversely, when the number of platelets is above 100,000 per $mm.^3$, the clot will retract well. Furthermore, there is a rough but distinct parallelism between the number of platelets and the quality of the clot. Thus, one method serves as a check on the other.

4. It should also be noted that the concentration of erythrocytes influences both the rate and the degree of clot retraction. When the erythrocyte mass is large, the plasma volume and consequently the total amount of fibrinogen is relatively small and the mass of erythrocytes limits the degree of retraction by the volume which it occupies in the clot. The converse prevails in anemia. Methods have been devised for the semiquantitative estimation of clot retraction which by the application of a formula adjust the results to a standard volume of packed red cells. In routine clinical hematology there is little need for this refinement.

REFERENCE

Budtz-Olsen, O E Clot Retraction Springfield, C C Thomas, 1951

Prothrombin Time

QUICK'S METHOD

Technic

1. Draw 4.5 ml of venous blood into a syringe. Immediately add the blood to a centrifuge tube containing 0.5 ml. of 0.1 M sodium

oxalate. Mix and centrifuge to obtain the plasma.

2. Place a small pyrex test tube (13 x 100 mm.) in a water bath at 37 C.

3 Add 0.1 ml. of oxalated plasma.

4. Add 0.1 ml of thromboplastin solution previously warmed to 37 C.

5. Allow the mixture to incubate for 1 minute.

6. By means of a short serologic pipet, forcibly blow 0.1 ml. of 0.02 M calcium chloride into the tube and click a stop watch simultaneously.

7. The tube is kept in the water bath and is constantly but gently shaken to within a few seconds of the expected clotting time. The tube is then removed from the water bath and held against a distant light source so that the plasma may be viewed through the bottom of the tube. The tube is gently tilted back and forth until the clot forms. The first sign of clotting is the end point.

8 By means of table 36, the clotting time in terms of per cent prothrombin can be computed.

Comments

1 Free flowing venous blood must be obtained without difficulty so as to avoid contamination of the specimen with tissue thromboplastin Undue exposure of the blood to air bubbles must be avoided and there must be no delay in adding the blood to the anticoagulant Since proaccelerin is labile, the plasma should be refrigerated promptly and the determination should be done within a few hours after the blood is obtained. The plasma cannot be frozen and then thawed because this destroys the proaccelerin, and a low level of prothrombin activity will result.

2 A potent thromboplastin must be used With each batch of thromboplastin solution the activity of a normal control plasma must be determined Ideally the value should fall between the limits of 11 to 13 seconds. Preparations giving a prothrombin time greater than 17 seconds are unsatisfactory.

3 0 025 M calcium chloride rather than 0.02 M may be required for clotting of plasma from patients with polycythemia or from patients receiving dicumarol therapy.

4 The calcium chloride must be discharged rapidly into the tube. It is a help in this regard to use a 1 ml. serologic pipet, gradu-

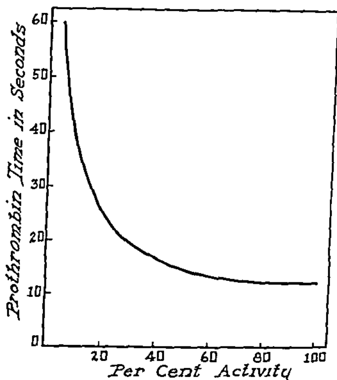


Fig 19—Curve relating prothrombin time in seconds to prothrombin activity in per cent of normal The plasma was diluted with saline

ated in tenths and hundreds of a ml and shortened to 17 cm. length.

5. The tube must be tilted gently. If it is shaken too vigorously, the initial tenuous fibrin mesh is apt to be broken and thus escape detection so that more time is required before further formation of fibrin occurs. This may introduce an appreciable error.

6. Heparin and antithrombin influence the coagulation time. If the patient is receiving heparin, the blood must be drawn at least 4 hours after the last injection of heparin or the value obtained will not be valid.

7. It must be pointed out that the curve relating the prothrombin time to prothrombin activity is hyperbolic (fig. 19). Therefore, it is completely erroneous to calculate the prothrombin activity by dividing the prothrombin time of the normal by that of the unknown.

8. From the configuration of the curve relating the prothrombin

time to the prothrombin activity (fig. 19), it is apparent that in the range of 25 to 100 per cent of normal activity a very small change in prothrombin time represents a substantial alteration in per cent activity. Therefore, conversion of an observed prothrombin time into prothrombin activity in this range is only an approximation at best. At lower levels of activity calculation by interpolation of the curve is more accurate.

9 Theoretically, for plasmas with 25 to 100 per cent of normal prothrombin activity it would be desirable to dilute the initial plasma sample. However, dilution with physiologic saline dilutes factors other than prothrombin (proaccelerin, proconvertin and fibrinogen) which are involved in the overall prothrombin activity.

Interpretation

1. This method is not specific for prothrombin. It measures the velocity of the entire clotting process except for the formation of thromboplastin A deficiency of prothrombin, proaccelerin, proconvertin, fibrinogen or a deficiency of a combination of these factors will result in a prolonged prothrombin time. In patients with hemophilia (AHG, PTC, or PTA deficiency) and in patients with thrombocytopenia the test is normal If the prothrombin time is prolonged, the presumptive tests for the specific deficiencies should be performed (p 122) A deficiency of fibrinogen may be suspected by the absence of a clot

2. Prothrombin activity may be measured both more accurately and more specifically by a modification of the above technic (p 119) In the modified technic a dilution of plasma is used, and the prothrombin time is measured on the more favorable portion of the curve A standard curve is prepared from normal human plasma diluted with plasma from which the prothrombin has been removed by absorption By this technic other factors which influence the prothrombin time are maintained at more optimal and constant concentrations

Reagents

Sodium oxalate (0.1 M)

Anhydrous sodium oxalate 1 34 Gm.
Distilled water to make 100 ml.

Calcium chloride (0.02 M)

Anhydrous calcium chloride 1.11 Gm.
Distilled water to make 500 ml.

Thromboplastin

Suspend 0.15 Gm. of lyophilized, acetone-extracted rabbit brain (Bacto-Thromboplastin, Difco Laboratories, Detroit 1, Michigan) in 4 ml. of a solution of physiologic saline. Add 0.05 ml. of 0.1 M sodium oxalate and incubate at 45 C. to 48 C. for 10 minutes with agitation every three minutes. Insert a 20 ml. volumetric pipet with a piece of cotton placed loosely over the tip into the suspension. By gentle suction draw the solution into the pipet. The thromboplastin solution so obtained should be used within a few hours. When stored at —20 C., the solution is stable for several months. Repeated freezing and thawing is not recommended, and therefore, it is convenient to store the solution in small aliquots.

TABLE 36—*Conversion of Prothrombin Time To Prothrombin Activity*

Prothrombin Time Seconds	Prothrombin Activity in Per Cent of Normal
11 — 12 5	100
13 5	60
15	50
17	40
19 5	30
22	25
21 — 36	20
37 — 40	10
55 — 65	5

REFERENCES

1. QUICK, A J. On the quantitative estimation of prothrombin Am J Clin Path *15* 560, 1945
2. —— The Hemorrhagic Diseases and the Physiology of Hemostasis, Baltimore, Charles C Thomas, 1942

MODIFIED METHOD

Technic

1. Draw 4.5 ml. of venous blood into a clean syringe. Immediately add the blood to a centrifuge tube containing 0.5 ml. of 0.1 M sodium oxalate Mix, and centrifuge to obtain the plasma.

2. Dilute 0.1 ml. of the test plasma with 0.9 ml. of prothrombin-free plasma (human or beef).

3. Place a test tube (10 x 75 mm.) containing 0.1 ml. of thromboplastin solution in a water bath at 37 C.

4. Add 0.1 ml. of the diluted oxalated plasma to the tube in the water bath Mix by tapping the test tube with the finger and allow he tube to stand in the water bath until the contents reach the temperature of the bath (about 10 minutes).

5. Draw 0.1 ml. of 0.025 M calcium chloride solution (kept in a 10 ml. test tube in the water bath) into a tuberculin syringe, and rapidly eject into the tube. A stop watch is started simultaneously.

6. The tube is kept in the water bath and is constantly but gently shaken to within a few seconds of the expected clotting time. The tube is then removed from the water bath and held against a distant light source so that the plasma may be viewed through the bottom of the tube The tube is gently tilted back and forth until the clot forms The first sign of clotting is the end point.

7. From the observed prothrombin time of the mixture, the percentage prothrombin is interpolated from the standard curve. This value, multiplied by 10 to correct for the 1:10 dilution, gives the prothrombin concentration of the undiluted plasma in per cent of normal

Preparation of the Standard Curve

1. Set up 9 small test tubes Pipet the following amounts of pooled normal human plasma into the tubes. tube 1, 1 ml.; tube 2, 0.8 ml., tube 3, 0.6 ml.; tube 4, 0.4 ml., tube 5, 0.3 ml.; tube 6, 0.2 ml., tube 7, 0.1 ml., tube 8, 0.05 ml., and tube 9, 0.01 ml.

2. Pipet the following amounts of prothrombin-free plasma into the tubes, tube 1, none, tube 2, 0.2 ml., tube 3, 0.4 ml.; tube 4, 0.6 ml., tube 5, 0.7 ml., tube 6, 0.8 ml.; tube 7, 0.9 ml.; tube 8, 0.95

ml.; and tube 9, 0.99 ml.

3. Mix the contents of the tubes.

4. Determine the prothrombin time on each mixture as outlined above. Plot the prothrombin times in seconds against the per cent prothrombin on arithmetic graph paper.

Comments

1. The comments concerning the prothrombin time of Quick are also pertinent to the modified technic.

2. It should be emphasized that a standard curve must be determined for each new batch of thromboplastin and prothrombin-free plasma.

3. The prothrombin-free plasma, if in the liquid state, must be *fresh since antithrombin develops rapidly and proaccelerin deteriorates rapidly.*

4. For extremely hypoproteinemic plasma 0.4 ml. of test plasma should be diluted with 0 6 ml. of prothrombin-free plasma

5. Duplicate determinations should agree within 0.5 second.

Interpretation

This method has the advantage over the method of Quick that with appropriate dilutions of the plasma the determinations are performed on the most favorable portion of the curve. *It is also* somewhat more specific for prothrombin deficiency since the prothrombin-free plasma, if properly prepared, supplies additional fibrinogen and proaccelerin. However, the barium sulfate removes proconvertin in addition to prothrombin, and therefore, the method may not be entirely specific. Furthermore, if the prothrombin-free plasma contains excess antithrombin an erroneously prolonged prothrombin time will result For the more specific estimation of prothrombin, the somewhat more laborious two-stage method of Warner, Brinkhous, and Smith[1] is recommended.

Reagents

Sodium oxalate (0.1 M) (See p. 117)

Calcium chloride (0.025 M)

Anhydrous calcium chloride 1.38 Gm.
Distilled water to make 500 ml.

Thromboplastin (See p. 118)

Pooled normal human plasma

Draw 4.5 ml of venous blood from each of 5 to 10 normal human subjects Immediately add each specimen to a centrifuge tube containing 0 5 ml. of 0.1 M sodium oxalate. Centrifuge the tubes at 2000 R.P.M for 10 minutes. Pool the plasma samples and place the pooled sample in the refrigerator at 4 C. The plasma must be used within several hours after the blood is obtained.

Prothrombin-free human plasma

Pooled normal human plasma, obtained as outlined above, is extracted with barium sulfate (C P., Baker). One hundred mg. of barium sulfate are added per ml. of pooled plasma. The mixture is incubated at 37 C. for 10 minutes with frequent agitation and is then centrifuged at 3000 R.P.M. for 30 minutes. The plasma is carefully separated from the sedimented barium sulfate It is important that all of the barium sulfate be removed from the supernatant plasma

The barium sulfate absorbed plasma should have a prothrombin time of no less than 180 seconds This preparation is unstable and if allowed to stand at room temperature for even an hour, the prothrombin time will be prolonged when it is added to pooled normal human serum. It can be stored in the liquid state at 4 C. for several hours, or at —20 C. in the dry state (lyophilized) for several months without loss of potency. Prothrombin-free beef plasma can be substituted for human plasma and can be purchased from the Difco Laboratories, Detroit, Michigan

REFERENCES

1 FROMMEYER, W B AND CORRIGAN H Determination of prothrombin by the dilution method Stability and activity of human and bovine prothrombin-free plasma J Lab & Clin Med *34* 1356, 1949

2 ALEXANDER, B, DEVRIES, A, AND GOLDSTEIN, R Prothrombin A critique of methods for its determination and their clinical significance New England J Med *240* 403, 1949

3 TOCANTINS L M, Ed The Coagulation of Blood Methods of Study New York Grune & Stratton, 1955 p 89

Presumptive Tests for Proaccelerin and Proconvertin Deficiencies

Technic

1. Draw 4.5 ml. of venous blood into a clean syringe. Immediately add the blood to a centrifuge tube containing 0.5 ml. of 0.1 M sodium oxalate. Mix, and centrifuge to obtain the plasma.
2. Mix the patient's plasma with the plasmas described under *Reagents.* The proportions are given in table 37. It is usually convenient to add 0.9 ml. of plasma A to 0.1 ml. of plasma B.
3. The prothrombin time (Quick) is determined on the mixtures as described previously (p. 114).

Comments

1. The tests outlined above can only be performed if the prothrombin time by Quick's method is sufficiently prolonged, as compared with a normal control, to allow detection of shortening of the clotting time.
2. In actual practice it is usually unnecessary to perform all of the tests listed in table 37.
3. When the patient's plasma exhibits only a *slightly prolonged* clotting time by Quick's method, the tests on the mixtures of 0.9 ml. of modified plasma and 0.1 ml. of the patient's plasma are usually more sensitive than the mixtures containing a preponderance of the patient's plasma.
4. Although these tests are not as sensitive as desired, they are usually adequate to detect and differentiate significant degrees of a deficiency of proaccelerin and proconvertin. They are of no value in quantitating the degree of deficiency.
5 Interpretation of the results is given in table 37

Reagents (See table 38)

Oxalated normal plasma

From a normal subject obtain blood as described under *Technic* above. This must be used within one hour after it is withdrawn. This preparation contains both proaccelerin and proconvertin.

TABLE 37—*Presumptive Tests for Proaccelerin and Proconvertin Deficiencies*

Reagent A 9 volumes	Reagent B 1 volume	Condition of Patient's Plasma if the Abnormal Clotting Time is Shortened	Not Shortened
Pt's plasma	Normal plasma		Contains an inhibitor
Pt's plasma	Absorbed plasma	Proaccelerin Def	Proconvertin Def.
Absorbed plasma	Pt's plasma	Contains Proconvertin	Proconvertin Def
Pt's plasma	Dicumarol plasma	Proaccelerin Def	Proconvertin Def.
Dicumarol plasma	Pt's plasma	Contains Proconvertin	Proconvertin Def
Pt's plasma	Aged plasma	Proconvertin Def.	Proaccelerin Def.
Aged plasma	Pt's plasma	Contains Proaccelerin	Proaccelerin Def
Pt's serum	Normal serum	Proconvertin Def.	Proaccelerin Def

Pt's = patient's, Def = deficient.

TABLE 38—*The Activity of the Coagulation Factors in Various Types of Plasma and in Serum*

Activity of	Reagent: Fresh Normal Plasma	Aged Normal Plasma	Normal Serum	Absorbed Normal Plasma
Prothrombin	+	+	O	O
Proaccelerin	+	O	O	+
Proconvertin	+	+	+	O
AHG	+	O	O	+
PTC	+	+	+	O
PTA	+	+	+	+

+, present, O, deficient

Serum

Allow blood to clot at 37 C. for two hours. Centrifuge and remove the serum Allow the serum to stand at room temperature for 24 hours Before use, decalcify the serum with one-fifth volume of 0 1 M sodium oxalate. This preparation is deficient in accelerin but not deficient in convertin.

Aged Plasma

Draw 4.5 ml of venous blood under sterile conditions Add the blood to a sterile centrifuge tube containing 0.5 ml of 0.1 M sodium

oxalate. Mix and centrifuge to obtain the plasma. Place the plasma in the refrigerator at 4 C. under sterile conditions for about one month. Then store the plasma in 1 ml. amounts in small tubes frozen at —20 C Under these conditions the plasma can be stored indefinitely. This preparation is deficient in proaccelerin. When used alone in the Quick test it should give a clotting time in excess of 60 seconds.

Absorbed Plasma (see p. 121)

This preparation is deficient in prothrombin and in proconvertin

Dicumarol Plasma

Obtain oxalated plasma from a patient who has received dicumarol for 3 to 4 days Although the clotting time by the Quick method will be prolonged, the plasma will contain a moderate amount of prothrombin but will be deficient in proconvertin. This preparation must be used within an hour after the blood is withdrawn

REFERENCE

OWEN, C. A, JR., MANN, F D, HURN, M H, AND STICKNEY, J M · Evaluation of disorders of blood coagulation in the clinical laboratory Am J. Clin Path 25 1417, 1955

Prothrombin Consumption Test

Technic

1. Remove serum from one of the two tubes used for the clotting time of whole blood (p 110) exactly one hour after the blood has clotted. If clot retraction is adequate, 0.1 ml of serum can be taken directly from the tube. If necessary, centrifuge the tube for three minutes at 1500 to 2000 R.P.M. in order to free the serum from the clot. Perform the test within 5 minutes after the serum is removed from the clot.

2. To a small test tube (13 x 100 ml) add 0.1 ml of thromboplastin solution previously warmed to 37 C, 0.1 ml of barium sulfate absorbed plasma, 0 1 ml. of 0 025 M calcium chloride and finally 0.1 ml. of the test serum The stop watch is started at the

time of the addition of the test serum and the clotting time is determined in the same manner as in the prothrombin time method (Quick) (p 114).

Comments

1. There are a great many modifications of the prothrombin consumption test. The above method is chosen because of its simplicity. It is as satisfactory as any of the simpler modifications.

2. All of the precautions concerning the withdrawal of blood for the clotting time (p. 111) apply also to the prothrombin consumption test. Likewise, the precautions concerning the performance of the prothrombin time by the Quick method also apply to this method.

3. The time intervals given above must be adhered to if reproducible results are to be obtained.

4 The barium sulfate absorbed plasma must be prothrombin-free.

5 It is always desirable to perform the test in duplicate or triplicate.

6 This test is considerably more sensitive in detecting a deficiency of thromboplastin formation than is the whole blood clotting time

Interpretation

1. With activation of thromboplastin, thrombin is formed and the prothrombin is utilized (consumed). Without activation of thromboplastin, thrombin does not form, and a large residual of prothrombin remains in the serum. This test is devised to measure the residual prothrombin activity in the serum and is an indirect measurement of thromboplastin activation.

2. Unfortunately, this simple one-stage method is not specific for the measurement of residual prothrombin activity. In addition to the unconverted prothrombin, the prothrombin time of serum depends upon (a) the thrombin formed during and after coagulation which has not been absorbed by fibrin or neutralized by the natural antithrombin, (b) the "accelerator effect" of the serum, (c) the concentration of proaccelerin not activated during coagu-

lation and (d) an adequate supply of fibrinogen. By allowing the blood to incubate for an hour after coagulation has occurred, most of the thrombin is neutralized by the natural antithrombins. This variable may be further controlled by incubating the separated serum at 37 C. in the presence of citrate. Neutralization of thrombin takes place more rapidly in the absence of a clot and the citrate prevents any further formation of thrombin. However, this additional step is usually not required for the routine diagnostic method By supplying adequate amounts of proaccelerin and fibrinogen in the barium sulfate absorbed plasma, these two variables are satisfactorily eliminated. However, the "accelerator effect" of serum is not eliminated. Fortunately, the serum accelerator effect does not mask defective prothrombin consumption because the two factors are present in an inverse relationship to one another. Thus, when there is a high residual activity of prothrombin, the amount of accelerator is low. When there is a low residual activity of prothrombin, the high level of accelerator has little effect since there is little prothrombin on which it can act. The result is that although there are many theoretically valid objections to the test, it is a very useful one for clinical purposes.

3 In hemophilia (AHG, PTC and PTA deficiencies) and in *patients with thrombocytopenia an abnormally high prothrombin* activity is found in the serum because of inadequate activation of thromboplastin.

4. By this method, a serum prothrombin time of 26 seconds or more is normal when a thromboplastin solution is used which gives a normal plasma prothrombin time of 12 to 17 seconds Values of less than 22 seconds may be considered abnormal

5 By dividing the plasma prothrombin time in seconds by the serum prothrombin time in seconds and multiplying the result by 100, a "prothrombin consumption index" can be derived. This gives the per cent of the total prothrombin activity which remains in the serum after coagulation Normally, this is less than 40 per cent.

Reagents

Thromboplastin (see p. 118)
Barium sulfate absorbed plasma (see p 121)
Calcium chloride (*0 025 M*) (see page p 121)

REFERENCES

1. Rosenthal, R. L.: Hemophilia and hemophilia like diseases caused by deficiencies in plasma thromboplastin factors: anti hemophilic globulin (AHG), plasma thromboplastin component (PTC) and plasma thromboplastin antecedent (PTA). Am J Med 17: 57, 1954.
2. Stefanini, M and Crosby, W. H: Serum prothrombin time, a composite effect. Am. J. Clin Path 20. 1026, 1950
3. —— and ——: The one stage prothrombin consumption Test. Blood 5. 964, 1950

Presumptive Tests for Differentiation of the Types of Hemophilia

Technic

1. Set up 8 clean test tubes (13 x 100 mm.) with an inside diameter of 11 mm. and a precalibrated mark on the side at a level of 1 1 ml. Label the tubes 1a, 1b, 2a, 2b, 3a, 3b, 4a, and 4b.
2. Add 0.1 ml. of 0.85 per cent saline to tubes 1a and 1b.
3. Add 0 1 ml. of barium sulfate absorbed normal plasma to tubes 2a and 2b.
4. Add 0 1 ml of fresh normal plasma to tubes 3a and 3b
5. Add 0 1 ml. of aged normal plasma to tubes 4a and 4b.
6. Draw venous blood from the patient and carefully fill each tube to the 1.1 ml mark
7. Determine the whole blood clotting time by the method outlined (p 110) on the 4 sets of tubes
8. After coagulation has occurred determine the serum prothrombin time (prothrombin consumption test, p 124) on one of each of the four pairs of tubes.

Comment

1. The comments concerning the precautions and sources of error in the determination of the clotting time (p. 111), plasma prothrombin time (p 115), and serum prothrombin time (p. 124), apply to the above method.
2. Aged normal plasma is deficient in AHG but contains adequate amounts of PTC and PTA (table 38). Barium sulfate ab-

sorbed plasma is deficient in PTC but contains adequate amount of AHG and PTA. Normal serum is deficient in AHG and ma be substituted for the aged normal plasma, or may be used i addition to it. However, serum may at times correct the clottin time of plasma-whole blood mixtures due to reasons other tha the presence of AHG.

3. At times barium sulfate absorption incompletely remove PTC from normal plasma. In tests on patients with a mild PTC deficiency, this may be a source of difficulty. The amount remain ing in the plasma may be enough to almost completely correct the defect. Reabsorption of the test plasma with barium sulfate, or use of plasma naturally deficient in PTC obtained from a severely affected person known to have the condition, obviates this difficulty.

4 Plasma from a patient with AHG deficiency will not correct the whole blood clotting time of a patient with AHG deficiency but will correct the clotting time of the blood from patients with either PTC or PTA deficiency. Plasma from a patient with PTC deficiency will not correct the whole blood clotting time of a patient with PTC deficiency but will correct the clotting abnormality of blood from patients with AHG or PTA deficiency. Plasma from a patient with PTA deficiency will correct the abnormality in the blood from patients with AHG or PTC deficiency, but not PTA deficiency. If fresh plasma is readily available from patients with these three types of coagulation disorders, such cross-correction tests are useful and may be substituted for the test outlined above, or used as a confirmatory procedure. However, they are not necessary to establish a diagnosis.

Interpretation

1. The results of the correction studies are summarized in table 39. If the abnormality is not corrected by fresh normal plasma, the presence of a circulating anticoagulant is suggested

2. The clotting time of the whole blood of patients with mild AHG, PTC, or PTA deficiency may be normal, or nearly normal. Under these conditions it is not possible to either detect the presence of an abnormality by the whole blood clotting time or to study its correction. Study of the serum prothrombin time is much more

sensitive than the whole blood clotting time, and therefore is more useful under these conditions. If the condition is severe and the whole blood clotting time is distinctly abnormal, it may be unnecessary to determine the serum prothrombin time of the plasma-whole blood mixtures.

3. The thromboplastin generation test is more sensitive than the tests outlined herein. It is recommended for the more specific entification of the type of disorder and for the diagnosis of the ilder types of these disorders.

TABLE 39—*Presumptive Tests for the Differentiation of the Types of Hemophilia*

Condition	Abnormality Corrected By			
	Fresh Normal Plasma	Barium Sulfate Absorbed Plasma	Aged Normal Plasma	Normal Serum
AHG Deficiency	Yes	Yes	No	No
PTC Deficiency	Yes	No	Yes	Yes
PTA Deficiency	Yes	Yes[1]	Yes[1]	Yes[1]

[1]Correction may be only partial

Reagents

Barium sulfate absorbed normal plasma (see p 121)
Fresh normal plasma (see p. 122)
Aged normal plasma (see p 123)

REFERENCES

1 ROSENTHAL, M C Deficiency in plasma thromboplastin component. II Its incidence in a hemophilic population Critique of methods for identification Am J Clin Path 24 910, 1954
2 ROSENTHAL, R L Hemophilia and hemophilia like diseases caused by deficiencies in plasma thromboplastin factors anti hemophilic globulin (AHG), plasma thromboplastin component (PTC) and plasma thromboplastin antecedent (PTA) Am J Med 17· 57, 1954
3 BIGGS, R, AND DOUGLAS, A S The thromboplastin generation test J Clin Path 6 23, 1953

Presumptive Tests for Fibrinogen Deficiency

Technic

1. If fibrinogen is absent, the blood will be incoagulable. A *simple test to differentiate afibrinogenemia from the hemophilic diseases* is to add several drops of thromboplastin solution to a small amount of incoagulable blood in a test tube. If the blood promptly coagulates, there is a deficiency of thromboplastin and not fibrinogen. If the blood does not coagulate after the addition of thromboplastin, fibrinogenopenia may be the cause.

2. Slowly heat a specimen of oxalated plasma to 60 C. If the plasma remains clear, a diagnosis of afibrinogenemia can be made since fibrinogen coagulates at 58 C.

Examinations Concerned with Hemolytic Disorders

In the study of a patient in whom hemolytic anemia is suspected, it is necessary to first determine whether or not a hemolytic anemia is present. In this regard, the importance of the blood smear must not be minimized.

The simple diagnostic features of a hemolytic anemia are listed in table 40. The methods for these determinations are described elsewhere in this manual.

It must be kept in mind that a "compensated" hemolytic disorder (hemolytic disease) may be present. In this event the patient will not be anemic but there will be evidences of an increased rate of erythrocyte production and destruction. On the other hand, in many patients the survival time of the erythrocytes may be shortened only moderately and the bone marrow may be unable to produce cells at a sufficient rate to compensate for the mild anemia In such patients there will be a mild anemia but the evidences of increased

TABLE 40—*Diagnostic Features of Hemolytic Anemia*

A *Evidences of Increased Rate of Erythrocyte Destruction*
1 Anemia
2 Hyperbilirubinemia (jaundice, icterus index elevated, indirect reacting bilirubin increased)
3 Increase in stool urobilinogen
4 Increase in urine urobilinogen
5 Hemoglobinemia
6 Methemoglobinemia
7 Hemoglobinuria
8 Hemosiderinuria

(Items 5–8: Intravascular hemolysis only)

B *Evidences of Increased Rate of Erythrocyte Production*
1 Reticulocytosis (macrocytosis)
2 Polychromatophilia
3 Anisocytosis
4 Poikilocytosis (spherocytosis, schistocytosis)
5 Nucleated red blood cells
6 Stippling
7 Thrombocytosis
8 Leukocytosis (neutrophilia)

production and destruction will be minimal or absent In these cases it may be necessary to measure the life-span of the erythrocytes and to measure the rate of erythrocyte production The technics developed for these purposes are not within the scope of a routine laboratory and, therefore, are not described in this manual.

Once it has been decided that one is dealing with a hemolytic disorder, then the various types of hemolytic disorders must be differentiated The hemolytic disorders comprise a large group of conditions which vary as to their cause, pathogenesis, severity, duration and treatment. A simplified classification is given in table 41 This classification, it must be admitted, is not satisfactory in all regards. To ascertain if a hemolytic process is due to an intracorpuscular or an extracorpuscular defect is not easily determined However, this classification effectively separates the hereditary from the acquired diseases and is helpful in understanding the underlying mechanisms

TABLE 41—*Classification of Hemolytic Anemia*

A. Intracorpuscular causes
   1. Hereditary spherocytosis (congenital hemolytic icterus)
   2. Hereditary non-spherocytic hemolytic anemia
   3. Hereditary elliptocytosis (ovalocytosis)
   4. Paroxysmal nocturnal hemoglobinuria
   5. Hereditary hemoglobinopathies—thalassemia, sickle cell, etc (see table 43)

B. Extracorpuscular causes
   1. Autoantibodies
      a. Warm autoantibody type
      b. Cold autoantibody type
         1) Associated with virus pneumonia
         2) *Paroxysmal cold hemoglobinuria*
   2. Isoantibodies
      anti-A, anti-B, anti-Rh, anti-Hr, anti-Kell, etc (Transfusion reactions, hemolytic disease of the newborn)
   3. Idiopathic (No demonstrable antibodies or primary disease)
   4. Secondary*
      a. Leukemia
      b. Lymphoma
      c. Carcinoma
      d. Liver disease
      e. *Collagen disorders*
      f. Myelofibrosis
      g. Sarcoid
      h. Ovarian tumors
      i. Thrombotic thrombocytopenic purpura
   5. Other causes
      a. Infectious agents
      b. Chemical agents
      c. Physical agents
      d. Vegetable poisons
      e. Animal poisons

*Autoantibodies may or may not be demonstrable

In the *intracorpuscular hemolytic anemias* the defect resides within the cells Thus, when erythrocytes from a patient with an intracorpuscular hemolytic anemia are transfused into a normal recipient, the cells survive for a shortened period of time just as they do in the patient. When erythrocytes from a normal subject are transfused into a patient with an intracorpuscular type of hemolytic anemia, the lifespan of the transfused cells is normal The hemolytic anemias due to an intracorpuscular defect are all inherited disorders, with the possible exception of paroxysmal nocturnal hemoglobinuria. Therefore, in this group a careful history of the presence of anemia, jaundice and splenomegaly in relatives

is of great importance. However, either a positive or a negative history may be very misleading and it is far better to examine all available relatives. As a screening procedure for the relatives it is quite adequate to determine the presence or absence of splenomegaly and hepatomegaly, determine the icterus index and volume of packed red cells, and make a *careful* examination of a *high quality* blood smear for evidences of increased blood production (table 40) and shape abnormalities of the erythrocytes (p. 74). In general, antibodies are absent from the blood of patients with hereditary hemolytic anemia (intracorpuscular). There are two exceptions to this rule. Patients who have received multiple blood transfusions may develop isoantibodies to the transfused cells. Secondly, autoantibodies may be present in the blood of patients with hereditary spherocytosis during a severe "hemolytic crisis."

The diagnostic tests of value in the differentiation of the intracorpuscular hemolytic anemias are listed in table 42

The clinical features of the hereditary hemoglobinopathies are summarized in table 43. In general the trait conditions (heterozy-

TABLE 42.—*Special Diagnostic Features of the Intracorpuscular Hemolytic Anemias*

Condition	RBC Morphology	Increased Osmotic Fragility	Acid Serum Test	Abnormal Hemoglobin Electrophoresis
Hereditary Spherocytosis	Spherocytes[1]	+	0	0
Hereditary Nonspherocytic	Normocytes	0	0	0
Hereditary Elliptocytosis	Elliptocytes	0	0	0
Paroxysmal Nocturnal Hemoglobinuria	Normocytes	0	+	0
Hereditary Hemoglobinopathies	Variable[2]	0[3]	0	+

[1]Spherocytes are not pathognomonic of hereditary spherocytosis. They may also be increased in some patients with acquired hemolytic anemia

[2]See table 43

[3]In the presence of target cells the osmotic fragility will be decreased

gosity) are not associated with a hemolytic anemia or morphologic abnormalities in the blood. The only exception to this rule is the presence of target cells in Hb C trait. Actually, the condition described as Hb H trait probably represents Thalassemia-Hb H. Hb H trait in the absence of the thalassemic gene has not yet been.

TABLE 43.—*The Hereditary Hemoglobinopathies*

Condition	Hb Type	Hb F %	Hemolytic Anemia	Splenomegaly	Sickling	Microcytosis	Hypochromia	Target Cells
None Adult	AA	<2	0	0	0	0	0	0
None Newborn	AF	50-85	0	0	0	0	0	0
Hb S Trait	AS	<2	0	0	+	0	0	±
Hb C Trait	AC	<2	0	0	0	0	0	+++
Hb D Trait	AD	<2	0	0	0	0	0	0
Hb E Trait	AE	<2	0	0	0	0	0	0
Hb G Trait	AG	<2	0	0	0	0	0	0
Hb H Trait	AH	0-4	++	+	0	+	+	+
Hb I Trait	AI	<2	0	0	0	0	0	0
Hb J Trait	AJ	<2	0	0	0	0	0	0
Hb K Trait	AK	<2	0	0	0	0	0	0
Sickle Cell Anemia	SS	2-24	++++	0	++	0	0	+
Homozygous C	CC	0-7	++	++	0	±	±	++++
Homozygous D	DD	<2	+	0	0	+	+	++
Homozygous E	EE	2-6	++	0	0	++	0	+++
Homozygous G	GG	<2	0	0	0	0	0	0
Sickle Cell—Hb C	SC	0-8	++	+	+	+	+	+++
Sickle Cell—Hb D	SD	<2	++	+	+	+	+	+
Sickle Cell—Hb G	SG	<2	0	0	+	0	0	±
Thalassemia Major	AF	10-90	+++	++++	0	+++	++	++
Thalassemia Minor	AA	<2	+	+	0	+	+	+
Thalassemia-Sickle	AS	1-17	++	+	+	+	+	+++
Thalassemia Hb C	AC	<2	+	0	0	+	±	+++
Thalassemia Hb E	AE	20-40	+++	+++	0	+++	++	+++
Thalassemia Hb G	G	<2	+	0	0	+	+	+
Thalassemia Hb H	AH	0-4	++	+	0	+	+	+
Thalassemia Hb S-Hb C	SC		++	+	+	+	+	+++
Thalassemia Hb S Hb G	SG	4	++	+	+	+	+	++

reported. As a general rule the homozygous state usually results in a definite hemolytic anemia of variable severity. An exception to this rule is Hb G disease. However, even though these patients have almost 100 per cent Hb G it has not yet been clearly demonstrated that the patients are truly homozygous for the Hb G gene. As another general rule, heterozygosity for two abnormal hemoglobin genes usually results in hemolytic anemia. However, the gene for Hb G, either alone or in various combinations, is not associated with a detectable physiologic disturbance. Another rule

is that if Hb S is present, either alone or in any combination, the sickle cell test will be positive.

Hb S and Hb C, with rare exceptions, are limited to Negroes and individuals of Negro ancestry The incidence of sickle cell trait in the American Negro is about 9 per cent, of sickle cell disease about 2 to 3 per cent, and of Hb C trait about 3 per cent. Hb D, however, has now been observed in Negroes, Caucasians, Algerians, and Indians. The incidence of Hb D trait in American Negroes is about 0 4 per cent Hb E is most prevalent in Oriental peoples descended from the inhabitants of southeast Asia. The incidence of Hb E trait in Thais is about 13 per cent and of Hb E disease about 0.3 per cent. The other hemoglobins have been detected in too few people for evaluation.

The defect in the *extracorpuscular hemolytic anemias* lies in the environment of the cells and not within the cells themselves. Thus, when erythrocytes from a patient with an extracorpuscular type of hemolytic anemia are removed from their unfavorable environment and are transfused into a normal recipient, they survive for a normal period of time On the other hand, when erythrocytes from a normal subject are transfused into a patient with an extracorpuscular type of anemia, the transfused cells are eliminated from the circulation more rapidly than normally. Insofar as is now known the extracorpuscular hemolytic anemias are acquired diseases and are not hereditary

The further classification of the extracorpuscular hemolytic anemias is difficult and it is far from satisfactory. Most are associated with detectable autoantibodies, some with isoantibodies, and in some no antibodies can be demonstrated In those with demonstrable antibodies the anemia is "idiopathic" because it has not been unequivocally demonstrated that the antibodies measured in vitro are the cause of, or related to, the excessive rate of blood destruction in vivo Even if this be the case, why antibodies to one's own cells develop is not at all understood Hemolytic anemia may be observed in association with other diseases (secondary hemolytic anemia) In such patients antibodies may or may not be detectable Finally, hemolytic anemia may be caused by chemical agents (drugs), infectious agents (bacteremia, viremia, bartonella,

malaria, etc.), physical agents (burns), vegetable poisons (fava beans, etc.) and animal poisons (snake venoms).

The nomenclature of antibodies is rather confusing to the novice and, therefore, the definitions which follow may be helpful.

Agglutinins are antibodies which cause *agglutination* of red cells The antigen of the red cell is called an *agglutinogen*. Complement is not necessary for agglutination to occur.

Hemolysins are antibodies which cause hemolysis of red cells in the presence of complement.

Autoantibodies are antibodies which are capable of acting on the patient's own corpuscles.

Isoantibodies are antibodies which are capable of acting on corpuscles from normal subjects. Isoantibodies may be "non-specific" i.e., they act on erythrocytes independently of their blood group. They may be "specific"; i.e., they act only on red cells of a certain blood group.

"Complete" antibodies are antibodies which are capable of causing agglutination or hemolysis in the presence of saline.

"Incomplete" antibodies can be detected in four ways: by the antiglobulin (Coombs') test, by means of trypsinized corpuscles, by titration in an albumin medium rather than a saline media, and by titration in the presence of large anisometric molecules.

Warm antibodies are antibodies which act maximally at 37 C. They act as incomplete antibodies. They occasionally act as complete agglutinating antibodies and rarely bring about hemolysis in saline media.

Cold antibodies are antibodies which are only slightly active or completely inactive at 37 C. and are markedly potentiated by cold. Cold antibodies act as "complete" agglutinating antibodies and under certain circumstances may cause hemolysis They may also act as "incomplete" antibodies

Dacie has divided the autoimmune hemolytic anemias *into two* types. those associated with a warm antibody, *and* those associated with a cold antibody (table 44). There are two rather distinct types of cold antibodies

In the *warm antibody type* there is an incomplete autoantibody. The optimum temperature for the indirect globulin test is 37 C., and the antibody titer is *little affected by acidification or by* heating the serum Complete warm agglutinins and hemolysins are

extremely rare. The complete cold agglutination titer may be between 1:2 and 1:512. Complete cold hemolysins cannot be demonstrated at acid pH.

The direct antiglobulin test is positive during periods of active hemolysis and is frequently positive during remissions. The indirect globulin test is positive in about two-thirds of the patients in the presence of active disease but is usually not positive during remissions.

In the *cold antibody type* there is a complete autoantibody as demonstrated by a cold agglutinin titer between 1:512 and 1:500,000 at 2 C. A complete cold hemolysin is frequently demonstrable (acid pH, 20 C), particularly in patients with an extremely elevated cold glutinin titer Complete warm agglutinins and hemolysins are t present However, the cold agglutinin is very commonly active room temperatures and if the blood is allowed to cool to room mperature in the syringe, agglutination may occur and be misaken for a warm agglutinin The direct antiglobulin test is positive luring periods of active hemolysis. The indirect antiglobulin test is usually positive at 20 C. The titer is enhanced by acidification (pH 6.5) but is completely inhibited by heating the serum This cold antibody type of hemolytic anemia is frequently but not always associated with virus pneumonia. Raynaud's syndrome may or may not be present

TABLE 41—*Autoimmune Hemolytic Anemia*

	Type of Antibody	
	Warm	*Cold*
Direct antiglobulin test	Positive	Positive
Indirect antiglobulin test	Positive	Positive
Optimal temperature	37 C	20 C
Effect of acidification	Little	Increases
Inhibition due to heating serum	Little	Complete
Complete cold agglutinin titer	2 — 512	512 — 500,000
Complete cold hemolysin (20 C, pH 6.5)	Absent	Present
Complete warm agglutinin	Rare	Absent
Complete warm hemolysin	Rare	Absent

A second and distinct type of cold autoantibody is observed in patients wi... rare disease, *paroxysmal cold hemoglobinuria* In

this condition the cold autoantibody, known as the *Donath-Landsteiner antibody*, is a "complete" hemolysin. The distinctive and remarkable feature of this antibody is that fixation on to the erythrocytes takes place only in the cold, and hemolysis occurs in the presence of complement after warming. The optimal pH for this antibody is 7.0 to 8.0. It also causes agglutination as well as strong sensitization to antiglobulin serum.

The type of autoantibody present can be differentiated by the tests outlined herein. These tests have been largely adapted from procedures described by J. V. Dacie, *The Haemolytic Anaemias, Congenital and Acquired*, New York, Grune & Stratton, 1954. This is a very excellent, comprehensive summary of the hemolytic anemias and the student is referred to this book for more elaborate and specific studies than are outlined herein.

Direct Antiglobulin (Coombs') Test for Incomplete Antibodies

Technic

1. Draw 10 ml. of the patient's blood into a syringe previously warmed to 37 C. Place the blood in a centrifuge tube (15 ml.) previously warmed to 37 C. and allow the blood to clot undisturbed at 37 C. in a water bath. Centrifuge at 2000 R.P.M. for 10 minutes. Draw off the serum and store it in a test tube immersed in a water bath (37 C.). Disrupt the clot and remove the fibrin portion. Fill the centrifuge tube with physiologic saline (0.85 Gm /100 ml) previously warmed to 37 C Centrifuge at 1000 R.P.M. for 2 minutes Draw off and discard the saline. Repeat the washing procedure two more times.

2. Transfer 0.2 ml of washed packed cells to a clean centrifuge tube (15 ml.). Fill the tube with saline and wash the cells repeatedly with saline until spontaneous agglutination has been eliminated. The cell button should resuspend easily without gross clumping.

3. Prepare a 2 per cent cell suspension by the addition of 10 ml. of physiologic saline

4. Place 0.2 ml of the 2 per cent cell suspension in a clean test tube (10 x 75 mm.).

5. Add 0.2 ml. of anti-human globulin (Coombs') serum.
6. Incubate for 37 C. for 30 minutes.
7. Centrifuge at 500 R.P.M. for one minute and read for agglutination. This is done by gently rotating the tube several times with a very slight wrist motion until the cells are stirred from the bottom of the tube. A bright source of visible light or a white background assists in reading the tubes. The degree of agglutination is graded as follows 1+ agglutination, a weak reaction unquestionably different from the control, 2+, many small clumps with clear pinkness of the surrounding fluid; 3+, large clumps with clear surrounding fluid; 4+, massive agglutination of all or nearly all cells.
8 A control test of the specificity of the antiglobulin serum is performed simultaneously by the use of fresh, unsensitized washed erythrocytes. A second control on the sensitivity of the test is performed by the use of cells sensitized in anti-D serum. Such cells can be prepared by adding 0.2 ml. of a 2 per cent suspension of washed normal D-positive (RH_o) cells to 0.2 ml of incomplete anti-D serum. The suspension is allowed to stand at 37 C. for 30 minutes and is then centrifuged at 1000 R P M. for 2 minutes The cells are washed three times with saline. Two-tenths ml of saline is added to the packed cells and the test is carried out as outlined above

Interpretation

1 The control test with the sensitized cells must be positive, and the control test with the normal unsensitized cells must be negative in order for the test to be valid.

2 In most patients with acquired hemolytic anemia due to autoantibodies, the direct antiglobulin (Coombs') test will be positive The test is negative in patients with hemolytic anemia due to an intracorpuscular cause with two exceptions. After multiple transfusions, sensitized red cells may be present. In a few patients with hereditary spherocytosis in hemolytic crisis, autosensitization may be superimposed upon the original congenital disease.

3 Unfortunately, it cannot be assumed that a direct antiglobulin test necessarily indicates that the patient is suffering from autoimmune hemolytic anemia or, if the test is negative, that the patient does not have such an anemia Both false positive and false negative

reactions can and do occur. False positive reactions may result from (a) sensitization occurring in vitro, or (b) from a "nonspecific reaction." False negative reactions may result from (a) the use of relatively impotent antiglobulin serum, (b) insufficient removal of the plasma or serum from the erythrocytes, or (c) the use of an inappropriate dilution of the antiserum.

Comments

1. Satisfactory antiglobulin serum can be purchased from commercial sources.* Care should be taken to purchase a serum that bears a National Institutes of Health license number. This certifies that the serum meets the minimal requirements of the National Institutes of Health, namely: "(a) Anti-human serum shall give 1+ agglutination of red cells exposed to the greatest dilution of the anti-Rh_o (Anti-D) serum which will react with those cells in albumin or comparable diluent, (b) the finished product when diluted at least 1:4 with saline shall give no less titer value for the anti-Rh_o (Anti-D) antibody than the undiluted anti-human serum, and (c) a protocol of a satisfactory potency test shall be submitted and must include negative control tests with undiluted anti-human serum and unsensitized group A and group B cells, and group O Rh negative cells exposed to a 1:16 dilution of anti-Rh_o serum."

Regardless of the source of antiglobulin serum each new batch must be tested for its specificity, i e , it must not agglutinate washed normal human erythrocytes. If it does so, the antiserum should be absorbed with human erythrocytes (group O) which have been washed 10 times with a large volume of saline. The suspension should stand for at least one hour at 4 C. If the antiserum still agglutinates normal cells, the absorption should be repeated with group A-B cells, or mixed A and B cells, which have been washed adequately with saline.

The potency of each batch of antiglobulin serum may vary considerably. Although the serum may be capable of agglutinating cells strongly sensitized in undiluted anti-D serum, it may be incapable of agglutinating cells weakly sensitized to anti-D or to other

*For example Scientific Products Division, American Hospital Supply Corp., Evanston, Ill , Ortho Pharmaceutical Corp , Raritan, N J , Certified Blood Donor Service, Inc , Jamaica, N Y

antibodies. Therefore, it is always advisable to titrate its ability to agglutinate erythrocytes sensitized to antibodies such as anti-D (p. 142) and the incomplete cold antibody which is usually demonstrable in normal serum.

Cold antibody-sensitized cells can be prepared by suspending one volume (50 per cent suspension) of washed group O erythrocytes (obtained from coagulated blood) in 9 volumes of fresh normal serum. The suspension is kept at 0 C. for two hours and then centrifuged and washed in three changes of saline warmed to 37 C. Serial twofold dilutions of the antiglobulin serum are made in saline, and the direct antiglobulin test is performed as outlined above. The end point is the highest dilution giving a 1+ agglutination.

The antiglobulin serum can be stored at 2 C. for a period of 1 year without loss of potency. Preservation is enhanced by storage at minus 10 C or lower.

2. The cells should be drawn on the day of the test. They may be obtained from oxalated, citrated, heparinized, coagulated or defibrinated whole blood. The procedure recommended under *Technic* for the separation of cells and serum permits separation of cells and serum under optimal conditions for all of the different antibody studies. If only the direct antiglobulin test is to be performed blood containing one of the above anticoagulants may be used and the cells need not be washed with *warm* saline.

3. If the clotted or defibrinated blood is chilled prior to the test, the reaction may be positive due to absorption of incomplete cold antibodies normally present in human sera This pitfall may be averted by collecting the blood as described under *Technic*.

4. "Nonspecific" reactions may occur rarely with blood from normal subjects and not uncommonly with blood from patients with hyperglobulinemia, even if the possibility of chilling in vitro has been excluded The "nonspecific" reactions are usually weak ones and are present only in high concentrations of a potent antiglobulin serum When such reactions are suspected the test should be carried out in several dilutions of antiserum.

5 Since the end point of the test is agglutination, the cells must be washed sufficiently to eliminate spontaneous agglutination. False positive agglutination may also result from high speed centrifugation

6. Human serum, if present, may neutralize the antiglobulin reagent and result in a false negative reaction. Therefore, extreme precautions about cleanliness of glassware and saline are necessary to avoid introduction into the test tube of even minute amounts of serum. The tubes must not be inverted for mixing since such a procedure involves the use of a potentially serum-contaminated finger or cork. The saline used for washing should be introduced *forcibly into the tube to disperse the red cells in the saline.*

7. The test can be quantitated by preparing serial fourfold dilutions of the antiglobulin serum in saline. This is done by placing 0.3 ml. of saline into each of 6 small clean test tubes. One-tenth ml. of antiglobulin serum is added to the first tube (1 in 4 dilution) and the contents are mixed by swirling the tube. One-tenth ml. of the 1 in 4 dilution is then added to the second tube *and the contents are mixed as before. Repeat this procedure in* each tube thereafter. Two-tenths ml. of each of the six dilutions (1:4, 1:16, 1:64, 1:256, 1:1024 and 1:4096) is then added to 0.2 ml. of the 2 per cent cell suspension and the test performed as outlined above. It should be kept in mind that the potency of a new batch of antiglobulin serum may vary considerably from the previous batch.

Determination of the Potency of the Antiglobulin (Coombs') Serum*

1. Because of the availability of the "blocking" or "incomplete" anti-D (anti-Rh_o) serum and D-positive (Rh_o) red cells as reagents, determination of the potency of anti-human serum is based on the ability of the serum to agglutinate red cells sensitized with minimal amounts of anti-D serum. An anti-D serum should be selected which contains anti-D antibodies which are not demonstrable with D-positive red cells in saline medium and which, when tested in a 20 per cent albumin solution, causes agglutination when the serum is diluted 1:64 or greater.

2. *In order to demonstrate the potency of the sensitizing anti-D serum*, prepare serial twofold dilutions (1:2 to 1:512)† of the

*Minimal requirements: *Anti-human serum for the antiglobulin test*, National Institutes of Health

†A dilution of 1:2 means 1+1

serum in 20 per cent albumin solution. Carry over of higher concentrations of serum to the tubes of greater dilution must be avoided by the use of separate pipets for each dilution

3. To each tube add an equal volume of a 2 per cent suspension of washed group O, D-positive (Rh_o) red cells suspended in 20 per cent albumin. The cells should be drawn on the day of the test as oxalated, citrated, clotted or defibrinated whole blood.

4. Incubate the tubes for 1 hour at 37 C. Centrifuge the tubes at 1000 R.P.M. for 2 minutes The end point is the greatest dilution giving a 1+ agglutination.

5. *In order to sensitize the red cells* prepare a 2 per cent suspension of washed group O, D-positive cells in physiologic saline (0.85 Gm./100 ml.).

6 Prepare serial twofold dilutions (1:2 to 1:512) in saline of the anti-D "incomplete antibody" serum in 2 ml. amounts.

7. Add 2 ml. of the red cell suspension to the various dilutions of anti-D serum and mix thoroughly.

8. Place the tubes in a water bath or incubator at 37 C. for 30 minutes.

9. Remove the tubes and wash the red cells by adding 10 ml. of saline to each tube. Centrifuge at 1000 R.P.M. for 2 minutes and withdraw the saline as completely as possible. Repeat this process until 3 washings have been made. Restore the washed cells to 2 ml. with saline.

10. *To test the potency of the antiglobulin serum*, prepare 6 tubes containing 1 ml. amounts of the antiglobulin serum diluted in saline in twofold steps from the undiluted preparation to 1:32.

11 Prepare 6 sets of 9 tubes each containing 0.1 ml. of sensitized red cell suspension. Each of the 6 sets of tubes will contain red cells exposed to decreasing amounts (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512) of anti-D antibody.

12 To each set of 9 tubes exposed to decreasing amounts of anti-D antibody, add 0.1 ml. of the antiglobulin serum. The first set of 9 tubes receives undiluted antiglobulin serum. The second set receives serum diluted 1 in 2 with saline; the third, serum diluted 1 in 4, etc

13 Mix the contents of each tube and place them in a water bath at 37 C for 30 minutes. Centrifuge at 1000 R.P.M. for 2 minutes.

14. The end point in each of the 6 sets of 9 tubes is the tube having the greatest dilution of anti-D antibody in which the cells show definite (1+) macroscopic agglutination.

15. A potent antiglobulin serum will give a 1+ agglutination of red cells exposed to the greatest dilution of anti-D serum which will react with those cells in albumin media.

16. Three control tubes, one with washed unsensitized group A cells, a second with washed unsensitized group B cells, and a third with group O Rh negative cells exposed to 1:16 dilution of anti-D serum should be negative.

Indirect Antiglobulin (Coombs') Test for Incomplete Antibodies

Technic

1. Serum is obtained from the patient as outlined for the direct antiglobulin test (p. 138). Acidified serum is prepared by adding 0.2 ml. of 0.25 N hydrochloric acid to 2.0 ml. of serum.

2. Normal erythrocytes, group 0 CDe/cDe (R_1R_2) and serum are obtained from blood allowed to coagulate at 37 C. The cells are then washed three times with large volumes (10.1) of saline. Finally, a 20 to 30 per cent suspension of the erythrocytes in physiologic saline is prepared in a graduated cylinder The cells must be obtained the day they are used and the suspension should not be made until it is required for use.

3. *Number 9 small test tubes (table 45)*

4. Place 0.3 ml. (6 drops) of the patient's unacidified serum in tubes 1, 2 and 4

5. Place 0.3 ml of the patient's acidified serum in tubes 5, 6 and 7

6 Place 0.3 ml of normal unacidified serum in tubes 3, 6 and 8.

7. Place 0.3 ml of normal acidified serum in tube 9

8. Place tubes 2 and 7 in a water bath or incubator at 56 C. for 30 minutes and then allow tube 2 to cool to 37 C and tube 7 to cool to room temperature (20 C.)

9. Place tubes 1 and 3 in a water bath at 37 C. to allow the serum to warm

10. Add one drop (0 05 ml.) of the suspension of erythrocytes to each tube. The cells added to tubes 1, 2 and 3 should have been previously warmed to 37 C.

11. Incubate the first three tubes at 37 C. for at least 2 hours Allow tubes 4 to 9 to stand at room temperature (20 C.) for a similar period of time.

12. Add warm (37 C.) saline to all of the tubes and centrifuge at 1000 R.P.M. for 2 minutes. Wash the cells two more times with warm saline.

13. Add 0.6 ml of physiologic saline to each tube and mix well

14. From each of the 9 tubes set up duplicate tubes each containing 0.2 ml. of cell suspension.

15. Add 0.2 ml. of anti-human globulin (Coombs') serum diluted 1 in 4 to each of the 9 tubes. Add 0 2 ml. of antiglobulin serum diluted 1 in 64 to the duplicate set of tubes.

16 Proceed as outlined for the direct antiglobulin test.

Interpretation

1 Warm antibodies are most active at 37 C. The cell suspension and serum must be warmed to this temperature before mixing and *the cell-serum suspension must be diluted in a large volume of* warm saline before removal from the water bath, as a preparation for centrifugation. Warm antibodies are enhanced little or not at all by an acid pH The absorption and fixation of warm antibodies is not influenced appreciably by the presence of complement and, therefore, the test can be carried out on heat-inactivated serum Frequently, warm antibodies are agglutinated most strongly in relatively highly diluted antiglobulin serum. Therefore, when dealing with an unknown antibody it is best to run the test with 1 in 4 and 1 in 64 dilutions of the antiglobulin serum.

2. Cold antibodies are most active at 20 C Tests *run at* 0 *to* 2 C may be falsely positive due to incomplete cold antibodies present in normal sera The sensitizing ability of pathologic incomplete cold antibodies is markedly enhanced by acidification and complement is required for fixation Cold antibodies fail to sensitize erythrocytes in serum which has been previously heated. In case the serum containing incomplete pathologic cold antibodies is deficient in complement it is advisable to add normal serum.

TABLE 45—*Indirect Antiglobulin Test*
Characteristic Reactions of Incomplete Warm and Cold Antibodies

Tube	Temperature °C	Serum	Acid	Agglutination Warm	Agglutination Cold
1	37	P	—	++++	0, +
2	37	P (Heated)	—	++	0
3	37	N	—	0	0
4	20	P	—	0	+
5	20	P	+	0	++++
6	20	P + N	+	0	++++
7	20	P (Heated)	+	0	0
8	20	N	—	0	0
9	20	N	+	0	0

P = patient, N = normal.

Erythrocytes sensitized by cold antibodies are more strongly agglutinated in concentrated antiglobulin sera than in diluted sera.

Comments

1. If a warm antibody is detected, its activity can be titrated Fourfold dilutions of the serum are made in saline This is done by placing 0.3 ml. of saline into each of 5 small test tubes. One-tenth ml of serum is added to the first tube (1 in 4 dilution) and the contents are mixed by swirling the tube. One-tenth ml. of the 1 in 4 dilution is then added to the second tube and the contents mixed as before Repeat this procedure in each tube thereafter. Two-tenths ml. of each of the five dilutions (1.4, 1·16, 1 64, 1·256, 1.1024) is then added to 0 2 ml. of a 2 per cent cell suspension of normal erythrocytes (group-O CDe cDE) which have been washed three times. After two hours at 37 C, the cells are washed three times with a large volume of saline, 0.2 ml. of antiglobulin serum (diluted 1,64) is added and the direct antiglobulin test is performed.

2. If a cold antibody is detected its potency can be titrated by making fourfold dilutions of the patient's acidified serum in acidified normal serum. The normal serum, prior to acidification, must

have been incubated three times for one hour at 2 C. with an equal volume of washed packed group-O erythrocytes in order to remove normal incomplete cold antibody. The antibody titration is carried out as for the titration of warm antibodies except that the sensitization is carried out at 20 C. and the cells are washed three times in warm (37 C.) saline.

3. If desired, the specificity of the warm or cold incomplete antibody can be determined by the use of a panel of normal erythrocytes of known blood groups and types. A manual on blood typing should be consulted for this purpose. Also, the thermal amplitude of the antibody can be determined by titrating the antibody at various temperatures such as 2 C., 10 C., 20 C., 30 C. and 37 C.

Trypsin Test for Incomplete Antibodies

Technic

1. Normal erythrocytes, group-O CDe/cDE are obtained from oxalated, citrated, or coagulated blood. Transfer 0.5 ml. of packed cells to a 15 ml. centrifuge tube and wash the cells three times with 10 to 15 ml. of saline. The patient's serum is obtained from blood allowed to clot at 37 C.

2. Two-tenths ml. of washed, packed, normal erythrocytes are added to one ml. of 0.1 per cent, buffered trypsin solution (p. 148). The mixture is incubated for one hour at 37 C. The "trypsinized" corpuscles are then washed in three changes of saline and finally 10 ml. of saline are added to make a 2 per cent cell suspension.

3. Fourfold dilutions of the patient's serum are made in saline so as to give serum dilutions ranging from undiluted serum to serum diluted 1 in 1024 (p. 142).

4. Number 7 small test tubes. Add 0.2 ml. of saline to the first tube, 0.2 ml. of undiluted serum to the second, and 0.2 ml. of each of the serum dilutions (1:4, 1:16, 1:64, 1:256 and 1:1024) to the remaining tubes. Place the tubes in a water bath at 37 C. to allow them to warm.

5. Warm the one per cent suspension of "trypsinized" corpuscles

to 37 C. and add 0.2 ml. (4 drops) to each tube.

6. Incubate all 7 tubes in a water bath for 2 hours at 37 C.

7. Examine the tubes for agglutination and grade the degree of agglutination from 0 to 4+ (p. 139).

8. Centrifuge the tubes at 1000 R.P.M. for 2 minutes and examine the supernatant solutions for hemolysis.

Comments

1. Because of the presence of a trypsin inhibitor in normal serum, the cells must be washed free of serum.

2. A nonspecific agglutination may occur with normal sera if the enzyme-treated cells and the sera are not warmed prior to incubation. A nonspecific agglutination may also occur with some normal sera if the concentration of the enzyme and the period of incubation of the cells with the enzyme are excessive. For this reason it is advisable to standardize the method with several different normal sera. If agglutination occurs, the period of incubation of the cells with the enzyme should be reduced to such a period of time that nonspecific agglutination does not occur with normal sera.

3. The titer of the antibody varies with the activity of the enzyme preparation, the enzyme concentration, cell concentration, pH, and time of incubation. The results will be comparable from one serum to another only if all of the above factors are kept constant.

4. The warm incomplete antibodies of acquired hemolytic anemia will almost always agglutinate trypsinized cells at 37 C. In some patients antibodies may be demonstrable using "trypsinized" cells and may not be demonstrable by the use of antiglobulin serum. Less commonly, these warm antibodies will sensitize normal corpuscles to antiglobulin serum and fail to agglutinate "trypsinized" red cells. Occasionally, warm hemolysins may be detected by the enzyme technic. "Trypsinized" cells are agglutinated by complete cold agglutinins more quickly and intensely than are normal corpuscles. The agglutinin titer is increased and the upper thermal limit for agglutination raised. Cold antibodies may hemolyze enzyme-treated cells even in unacidified serum.

Reagents

Trypsin Solution. A 1 per cent stock solution of crystalline trypsin

in dilute hydrochloric acid is made as follows:

Crystalline trypsin (Armour) 0.1 Gm.

Hydrochloric acid 0.05 N to make 10 ml.

This solution will keep for several months at 4 C. A dilute working solution is made from the concentrated stock solution as follows:

Trypsin solution (1 per cent) 1 ml.

Phosphate buffer (pH 7.7, 0.1 M) 9 ml.

The dilute (0.1 per cent) trypsin solution must be prepared just prior to use

The 0.1 M phosphate buffer (pH 7.7) is prepared by adding 90.5 ml. of a 1 63 per cent solution of anhydrous Na_2HPO_4 to 9 5 ml. of a 2 34 per cent solution of $NaH_2PO_4 2H_2O$.

Polyvinylpyrrolidone (PVP) Test for Incomplete Antibodies

Technic

1. Add 0.1 ml of whole blood or of packed red cells to 0.5 ml of a 5 per cent buffered solution of polyvinylpyrrolidone (PVP).

2. Mix and allow to stand at room temperature until settling is evident (about 15 min.).

3 Add 15 ml. of physiologic saline and invert the tube two or three times

4. Within several minutes after the saline dilution is made, the intensity of agglutination or rouleau formation is read microscopically.

5 The presence of short chains containing 2 to 4 red cells is scored as 1+, longer chains and occasional small clumps as 2+, many aggregated rouleau and numerous clumps as 3+, and coarse macroscopic clumps as 4+.

Comments

Normal unsensitized red cells form rouleau in 5 per cent PVP, but with sufficient dilution with saline the rouleau formation does not persist. On the other hand, the agglutination and rouleau forma-

tion of sensitized cells persist after dilution with saline. PVP is more sensitive as a developing agent than either 25 per cent albumin or Coombs' serum. The sensitivity and the simplicity of the PVP test recommend its use as a routine hematologic technic.

Reagents

Polyvinylpyrrolidone (PVP) Solution (5 per cent)

PVP 5 Gm.

Phosphate buffer to make 100 ml.

This solution may be kept in the refrigerator for several weeks. Polyvinylpyrrolidone (PVP), K-44, molecular weight of 160,000 can be obtained from Schenley Laboratories, Inc., New York, N. Y.

Phosphate buffer

M/15 Sorensen's phosphate buffer, pH 7.4, is prepared from sodium phosphate (Merck, reagent grade, anhydrous, dibasic sodium phosphate, Na_2HPO_4) and potassium phosphate (Merck, reagent grade, anhydrous, monobasic potassium phosphate, KH_2PO_4). *Dissolve 9.47 Gm. of sodium phosphate in distilled water* and make up to 1 liter. Dissolve 9.08 Gm of potassium phosphate in distilled water and make up to 1 liter. Add 80.8 ml. of the sodium phosphate solution to 19 2 ml of the potassium phosphate solution. These solutions may be kept for some weeks in pyrex glass in the refrigerator.

REFERENCE

JANDL, J A AND CASTLE, W B Agglutination of sensitized red cells by large anisometric molecules J Lab & Clin Med 47 669, 1956

Presumptive Tests for Complete Antibodies

Technic

1 Draw 10 ml of the patient's blood into a syringe previously warmed to 37 C. Place the blood in a centrifuge tube (15 ml) previously warmed to 37 C and allow the blood to clot undisturbed at 37 C. in a water bath. *Centrifuge at 2000 R.P.M. for 10 minutes.* Draw off the serum and store it in a test tube immersed in a water

bath at 37 C. Disrupt the clot and remove the fibrin portion. Fill the centrifuge tube with physiologic saline (0.85 Gm./100 ml.) previously warmed to 37 C. Centrifuge at 1000 R.P.M. for 5 minutes. Draw off and discard the saline. Repeat the washing procedure two times. Make a 50 per cent suspension of cells in physiologic saline by the use of a pipet.

2. Place 6 small test tubes in a water bath (37 C.) and number the tubes 1 through 6.

3. Add 0.5 ml. of serum to each tube.

4. Add 0.05 ml. of 0.2 N hydrochloric acid to tubes 3 and 4. Mix by rotating the tubes.

5. Add 0.05 ml. (one large drop) of the cell suspension to each of the 6 tubes

6. *Tube 1.* Centrifuge immediately at 1000 R.P.M. for 2 minutes and examine the supernate for hemolysis. Save this tube.

Tubes 2 and 3. Incubate at 37 C. for 1.5 hours. Examine the red cells macroscopically for agglutination. Centrifuge at 1000 R.P.M. for 2 minutes and examine the supernates for hemolysis.

Tube 4. Incubate at room temperature (15 C. to 20 C.) for 1.5 hours. Examine the red cells for agglutination. Centrifuge at 1000 R.P.M. for 2 minutes and examine the supernate for hemolysis

Tube 5. Chill in an ice bath or refrigerator for 1.5 hours. Examine for agglutination. Centrifuge immediately at 1000 R.P.M. for 2 minutes. Examine the supernate for hemolysis.

Tube 6. Chill in an ice bath or refrigerator for 30 minutes. Then, incubate the tube for 1 hour at 37 C. Centrifuge at 1000 R.P.M. for 2 minutes and examine the supernate for hemolysis.

Interpretation

Tube 1. Control tube. If the serum is hemolyzed at the start due to mechanical trauma to the blood there is no point in proceeding with the test. A fresh unhemolyzed serum must be obtained. If the patient has paroxysmal nocturnal hemo- [illegible]

beads, 3 to 4 mm in diameter per 10 ml of blood).

Tube 2. The presence of agglutination suggests a complete warm agglutinin. This type of antibody is rare and may easily be mistaken for the more common complete cold agglutinin if the blood is at

any time allowed to cool below 37 C. Agglutination must also be differentiated from rouleau formation. The absence of agglutination does not exclude the possibility of an "incomplete" warm agglutinin.

Hemolysis in this tube (compare with tube 1) suggests the presence of a complete warm hemolysin. Paroxysmal nocturnal hemoglobinuria (P.N.H.) cells may hemolyze without acidification of the serum. If a true complete warm hemolysin is present its activity should be enhanced at an acid pH (tube 3) and the definitive acid-serum test (p. 159) for P.N.H. should be negative. The absence of hemolysis in this tube does not exclude the presence of an "incomplete" warm hemolysin.

Tube 3. If agglutination is present in tube 2 it is likely to be present in this tube, and it has the same significance as outlined above.

Hemolysis in this tube (compare with tube 1) may be due to either a complete warm hemolysin (see above) or abnormal red cells (P N.H.) which are hemolyzed at an acid pH in normal serum If hemolysis is present in this tube, the definitive test for the diagnosis of P.N.H. must be performed (p. 159). If no hemolysis is present in this tube it is unnecessary to proceed further to rule out P.N.H.

Tube 4 If a cold agglutinin is present it may give a weak reaction in this tube. The optimal temperature for a cold agglutinin is 2 C. (tube 5) and in most sera the intensity of agglutination in strong concentrations of serum is reduced by acidification.

Hemolysis in this tube, particularly in the presence of cold agglutinins (tube 5) and in the absence of hemolysis in tube 3, is a positive presumptive test for a cold hemolysin. In this case the definitive test for cold hemolysins should be performed (p. 156) and the titer of cold agglutinins and hemolysins should be determined 154). If hemolysis is absent in this tube it is unnecessary to proceed to the more elaborate test

Tube 5. Agglutination in this tube after chilling indicates the presence of a cold agglutinin. If cold agglutinins are present the titer of activity should be determined (p. 154) The presence of a cold agglutinin in a low titer usually has no clinical significance. If agglutination is absent in this tube it is unnecessary to proceed to the more elaborate test

Even if a cold hemolysin is present (tube 1) it may not be demonstrated in this tube because the optimal temperature and pH

for the demonstration of a cold hemolysin is about 20 C. and a pH of 6.5 to 6.8. If the rare cold hemolysin of the Donath-Landsteiner type is present (tube 6) hemolysis should not be present in this tube if it is not allowed to rewarm after chilling.

Tube 6. Hemolysis after rewarming is presumably due to the rare cold hemolysin of the Donath-Landsteiner type. The definitive test (p. 158) should be carried out to establish the diagnosis of paroxysmal cold hemoglobinuria (P.C.H.). If no hemolysis occurs, the diagnosis of P.C.H. is excluded except for the highly improbable possibility that the serum is deficient in complement.

Comments

1. The degree of agglutination is graded as follows: 1+ agglutination, a weak reaction unquestionably different from the control; 2+, many small clumps with pinkness of the surrounding fluid; 3+, large clumps with clear surrounding fluid; 4+, massive agglutination of all or nearly all cells

2 The degree of hemolysis is graded as follows: 1+ hemolysis, pale red supernate which is definitely deeper in color than the control; 2+ and 3+, increasing degrees of redness of the supernates, 4+, complete hemolysis.

3 It must be realized that negative presumptive tests for "complete" autoantibodies do not rule out the diagnosis of autoimmune types of hemolytic anemia. The autoantibodies are far more frequently "incomplete" than "complete." Warm complete antibodies, paroxysmal cold hemoglobinuria, and paroxysmal nocturnal hemoglobinuria are extremely rare.

TABLE 46—*Characteristic Reactions Presumptive Tests for Complete Antibodies*

Tube	Acid	Temperature and Time	Agglutinin		Hemolysin		D L[1] Cold Hemolysin	P N H[2] Acid Test
			Warm	Cold	Warm	Cold		
1	0	37 C, 0 hr			0	0	0	±
2	0	37 C., 1.5 hr	++++	0	++	0	0	±
3	+	37 C, 1.5 hr	++++	0	++++	0	0	++++
4	+	20 C, 1.5 hr	±	+	++	++++	0	+++
5	0	2 C., 1.5 hr	0	++++	0	±	0	0
6	0	2 C., 0.5 hr						
		37 C 1 hr			0		++++	0

[1]Donath Landsteiner
[2]Paroxysmal Nocturnal Hemoglobinuria

Complete Cold Agglutinins

Technic

1. Draw 10 ml of the patient's blood into a syringe previously warmed to 37 C. Place the blood in a centrifuge tube (15 ml.) previously warmed to 37 C. and allow the blood to clot undisturbed at 37 C. in a water bath. Centrifuge at 2000 R.P.M for 10 minutes. Draw off the serum and store it in a test tube at room temperature. Disrupt the clot and remove the fibrin portion. Fill the centrifuge tube with physiologic saline (0 85 Gm./100 ml.) previously warmed to 37 C. Centrifuge at 1000 R.P.M. for 5 minutes. Draw off and discard the saline. Repeat the washing procedure three times. Make a 1 per cent suspension of cells in physiologic saline. This is done by adding, for example, 0.5 ml. of washed cells to 50 ml. saline in a graduated cylinder. Care should be taken to deliver the cells directly into the saline. It is advisable to place about one ml. of the cell suspension in a refrigerator (0 C. to 5 C.) for one hour while the tubes are being set up. Before use, the cells are then checked macroscopically to be certain that they have been sufficiently washed free of serum to prevent agglutination. If the cells are agglutinated they must be washed until agglutination no longer occurs in the cold.

2. Place 17 small, clean test tubes in a test tube rack.

3. Omitting tube 1, place 0 5 ml. of physiologic saline in each tube thereafter

4. Add 0 5 ml. of serum to tubes 1 and 2

5. Mix the serum and saline in tube 2 by repeatedly drawing up 0.5 ml amounts into a 1 ml pipet. Transfer 0 5 ml of this serum-saline mixture to tube 3 and mix as in tube 2. Repeat this procedure in each tube thereafter. Remove and discard 0 5 ml. of the mixture from the next to the last tube (No. 16) Do not add serum-saline mixture to the last tube (No 17). A final quantity of 0.5 ml of solution remains in each tube.

6 Add 0.5 ml of the well stirred red cell suspension to each tube. Mix each tube by gentle rotation.

7. Each tube now contains 1 ml. of solution. The last tube serves as a control on the adequacy of the washing of the red cells and should show no agglutination. Starting with tube 1 the dilutions

are 1:2, (2) 1:4, (3) 1:8, (4) 1:16, (5) 1:32, (6) 1:64, (7) 1:128, (8) 1:256, (9) 1:512, (10) 1:1000, (11) 1:2000, (12) 1:4000, (13) 1:8000, (14) 1:16,000, (15) 1:32,000, (16) 1:64,000, (17) control. Under certa... desirable to se...

8. Incu... e temperature (15 C. to 22 C.) a... the tubes macroscopically for agglutination. A hand lens may be of value.

9. Place the tubes in a refrigerator (2 C. to 5 C.) for 18 to 24 hours. Remove the rack and read the tubes immediately before they are allowed to warm up.

10. Resuspend the cells and place the rack in a water bath at 37 C. for 1 hour Examine for agglutination.

Interpretation

1 The tubes are gently rotated several times with a very slight wrist motion until the cells are stirred from the bottom of the tube A bright source of visible light or a white background assists in reading of the tubes. A magnifying hand lens may be used but is not required The highest dilution in which any degree of agglutination is observed is taken as the titer. The degree of agglutination is graded as follows: 1+ agglutination, a weak reaction unquestionably different from the control; 2+, many small clumps with pinkness of the surrounding fluid; 3+, large clumps with clear surrounding fluid, 4+, massive agglutination of all or nearly all cells

2 Titers of less than 1:32 are of no significance. In patients with acquired hemolytic anemia associated with a cold antibody the titer is usually between 1:1000 and 1:500,000. In patients with acquired hemolytic anemia associated with a warm antibody the titer is usually less than 1:64 but rarely may be as high as 1:512

Comments

1. Group-O cells may be used in place of the patient's cells The titer is a little different with group-O cells as compared with the patient's own cells The titer may vary somewhat with group-O cells from different individuals

2. Oxalated, citrated or heparinized blood may be used in place of clotted blood since complement is not essential for agglutination to occur.

3. The cells used should be fresh. The serum may be stored, without loss of potency, for several days at refrigerator temperatures or for longer periods at —20 C.

4. Care must be taken in the pipeting of serum dilutions, because small amounts of undiluted serum may line the top of the pipet and may be subsequently washed into the serial dilutions.

5. The cold agglutination of red cells is known as a "soft" aggregate and as such is easily dispersed by heat and mechanical agitation Therefore, the tubes must be read immediately after removal from the refrigerator and the aggregates must be dispersed by gentle rotation rather than by shaking.

6 The thermal amplitude of the cold agglutinins can be determined as described above by preparing 6 sets of 17 tubes. One set of tubes is incubated at each of the following temperatures: 2 C., 10 C., 15 C., 22 C., and 37 C. The complete cold antibodies are characteristically most active at 0 C. to 5 C The titer is reduced as the temperature is raised to 25 C. and agglutination is abolished at temperatures above 28 C. to 32 C.

Complete Cold Hemolysins

Technic

1. Draw 10 ml of the patient's blood into a syringe previously warmed to 37 C Place the blood in a centrifuge tube (15 ml.) previously warmed to 37 C. and allow the blood to clot at 37 C. in a water bath. Centrifuge at 2000 R.P.M. for 10 minutes. Draw off the serum and store it at room temperature (15 C to 22 C.). Disrupt the clot and remove the fibrin portion. Fill the centrifuge tube with physiologic saline (0.85 Gm./100 ml.) previously warmed to 37 C. Centrifuge at 1000 R P.M. for 5 minutes. Repeat the washing procedure two times Make a 50 per cent suspension of cells in physiologic saline by the use of a pipet

2. Obtain serum from fresh normal blood (group-O) allowed to clot undisturbed at 37 C.

3. Place 4 small test tubes in a water bath at 37 C. Number the tubes.

4. As outlined in table 47, add 0.5 ml. of the patient's serum to tubes 1 and 3; 0.5 ml. of normal serum to tube 4; 0.25 ml of the patient's serum and 0.25 ml. of normal serum to tube 2.

5. Add 0.05 ml. of 0.2 N (or 0.25 N) hydrochloric acid to tubes 1, 2 and 4 and mix.

6 Add 0 05 ml. (one large drop) of the patient's cells to each tube. Care should be taken to deliver the cell suspension directly into the serum

7. Incubate the first 4 tubes at room temperature (15 C to 22 C.) for two hours. Incubate tube 5 at 37 C. for 2 hours.

8. Centrifuge the tubes at 1000 R.P.M. for 2 minutes and examine the supernates for hemolysis.

Interpretation

1. The degree of hemolysis is graded as follows: 1+ hemolysis, pale red supernate which is definitely deeper in color than the control, 2+ and 3+ increasing degrees of redness; 4+, complete hemolysis.

2. The typical hemolytic reaction of a complete cold hemolysin is given in table 47. Hemolysis characteristically occurs only in the tubes containing the patient's acidified serum (tubes 1 and 2) which have been incubated at 20 C.

3 A temperature of 20 C. is optimal for this type of hemolysis There is no advantage in incubating at 37 C. after sensitizing at 20 C It is not only unnecessary but undesirable to chill the cells to 2 C. and then warm to 37 C. Lysis may be diminished at 2 C. as compared with 20 C.

4 Normal serum is added to tube 2 to allow for the possibility that the patient's serum is deficient in complement.

5 The titer of cold hemolysins may be determined by making serial fourfold dilutions of the patient's serum in acidified (10 per cent by volume of 0 25 N hydrochloric acid) fresh normal serum, and adding to the serum dilutions equal volumes of a 2 per cent suspension of either the patient's cells or normal (group-O) cells The tubes should be incubated at 20 C for 2 hours

TABLE 47.—*Characteristic Reactions of Complete Cold Hemolysins*

Tube	Temperature	Cells 0.05 ml.	Serum 0.5 ml.	Acid 0.05 ml.	Hemolysis
1	20 C.	P	P	+	+
2	20 C.	P	P + N	+	+
3	20 C.	P	P	0	0
4	20 C.	P	N	+	0
5	37 C.	P	P	+	0

P = patient; N = normal

Donath-Landsteiner Test

Technic

1. Draw 10 ml. of the patient's blood into a syringe previously warmed to 37 C Place the blood in a centrifuge tube (15 ml.) previously warmed to 37 C. and allow the blood to clot. Centrifuge at 2000 R.P.M. for 10 minutes. Draw off the serum and store it in a test tube immersed in a water bath (37 C.). Disrupt the clot and remove the fibrin portion. Fill the centrifuge tube with physiologic saline previously warmed to 37 C. Centrifuge at 1000 R.P.M. for 5 minutes. Draw off and discard the saline Repeat the washing procedure two times. Make a 50 per cent suspension of cells in physiologic saline by the use of a pipet.

2. Process 10 ml. of normal blood (group-O) in the manner outlined under 1.

3. Place 8 small test tubes in a water bath at 37 C. Number the tubes.

4. As outlined in table 48, add 0.5 ml. of the patient's serum to tubes 1, 3, 4 and 6; 0.5 ml. of normal serum to tubes 5, 7, and 8; 0.25 ml of the patient's serum and 0.25 ml. of normal serum to tube 2.

5. Place tube 3 in a water bath or incubator at 56 C. for 30 minutes and then return it to the 37 C. water bath

6. Add 0.05 ml. (one large drop) of cell suspension of the patient's cells to tubes 1, 2, 3, 5, 6, and 7.

7. Add 0.05 ml. (one large drop) of the suspension of normal cells to tubes 4 and 8.

8. Chill tubes 1 through 5 in an ice bath for 30 minutes and then incubate the tubes at 37 C. for one hour. Centrifuge at 1000 R.P.M. for 2 minutes and inspect the supernates for hemolysis.

9. Leave tubes 6, 7 and 8 at 37 C. for one hour. Centrifuge at 1000 R.P.M. for 2 minutes and inspect the supernates for hemolysis.

Interpretation

Hemolysis in tubes 1, 2, and 4 and not in tubes 3, 5, 6, 7 and 8 is diagnostic of paroxysmal cold hemoglobinuria.

TABLE 48.—*Donath Landsteiner Test*

Tube	Serum 0.5 ml.	Cells 0.05 ml	Temperature and Time	Hemolysis
1	P	P	2 C., ½ hr., 37 C., 1 hr	+
2	P + N	P	2 C., ½ hr., 37 C., 1 hr	+
3	P (Heated)	P	2 C., ½ hr., 37 C., 1 hr	0
4	P	N	2 C., ½ hr.; 37 C., 1 hr	+
5	N	P	2 C., ½ hr., 37 C., 1 hr.	0
6	P	P	37 C., 1 hr	0
7	N	P	37 C., 1 hr.	0
8	N	N	37 C., 1 hr	0

P = patient, N = normal

Acid-Serum Test for Paroxysmal Nocturnal Hemoglobinuria

Technic

1. Defibrinate 10 ml. of the patient's blood in a small Erlenmeyer flask containing 10 glass beads (3 to 4 mm. diameter) by gently rotating the flask until the hum of the beads on the glass is no longer audible. Decant the blood and separate the cells and serum by centrifugation (2000 R.P.M. for 5 minutes). Wash the cells three times with at least an equal volume of physiologic saline each time. Prepare a 50 per cent suspension of the patient's washed erythrocytes in physiologic saline.

2. Process 10 ml. of normal blood (group-O) in the manner outlined under 1.*

3. Place 7 small test tubes in a water bath at 37 C. and number the tubes.

4. As outlined in table 49, place 0.5 ml. of the patient's serum in tubes 1, 2, 6 and 7, and 0.5 ml. of normal serum in tubes 3, 4 and 5

5 Place tube 3 in a water bath or incubator at 56 C. for 30 minutes and then return it to the 37 C. water bath.

6. Add 0.05 ml. of 0.2 N hydrochloric acid to tubes 2, 3, 5 and 7.

7. Add 0.05 ml. (one large drop) of the suspension of the patient's cells to tubes 1 through 5 and a similar volume of a suspension of normal cells to tubes 6 and 7.

8. *Incubate all of the tubes at 37 C. for 1 hour.*

9. Centrifuge the tubes at 1000 R.P.M. for 2 minutes.

10. Examine the supernates for hemolysis.

Interpretation

1. *The hemolytic reaction outlined in table 49 establishes the* diagnosis of paroxysmal nocturnal hemoglobinuria. A trace of hemolysis may be present in tubes 1, 3 and 4

2. Hemolysis in tubes 2 and 7 (or 1, 2, 6 and 7) and not in tube 5 indicates the presence of a warm hemolysin

3. Markedly spherocytic erythrocytes may undergo lysis in acidified serum *(tubes 2, 3 and 5). However, P N H. cells do not undergo* lysis in heated serum whereas the lysis of spherocytes is unaffected by heating the serum.

TABLE 49.—*Acid Serum Test for P N H*

Tube	Cells *0 05 ml*	Serum *0 5 ml*	Acid *0 05 ml*	Hemolysis
1	P	P	0	0
2	P	P	+	+
3	P	N (Heated)	+	0
4	P	N	0	0
5	P	N	+	+
6	N	P	0	0
7	N	P	+	0

P = patient, N = normal

*The normal cells and serum may be obtained from clotted blood

Presumptive Osmotic Fragility

Technic

1. Pipet 0.1 ml. of oxalated venous blood into each of two test tubes.
2. Pipet 1.0 ml. of an 0.85 per cent solution of sodium chloride into the first tube.
3. Pipet 1.0 ml. of an 0.50 per cent solution of sodium chloride into the second tube.
4. Mix and centrifuge.
5. If hemolysis occurs in the second tube, the osmotic fragility of the erythrocytes is probably increased.

Comments

1. Blood obtained from a normal individual should be run as a control.
2. The above test is a crude screening test and if it is positive, the osmotic fragility should be studied by the quantitative method.

Quantitative Osmotic Fragility

Technic

1. Fifteen to 20 ml. of blood are drawn under aseptic conditions and placed in a sterile Erlenmeyer flask containing 15 small glass beads (3 to 4 mm. in diameter) and rotated gently until the hum of the beads on the glass is no longer audible. Approximately 2 ml of blood are then pipetted under sterile conditions into each of 4 sterile screw-capped vials and set aside for the incubated osmotic fragility and for the autohemolysis test. The remainder of the blood is used for the determination of the osmotic fragility prior to incubation. If osmotic fragility and autohemolysis after incubation are not to be determined, a smaller volume of blood may be drawn and aseptic precautions need not be observed. However, it is frequently desirable to determine the osmotic fragility both before and after incubation and also to do the autohemolysis test (p. 164).

2. Five ml. of each of the following solutions are placed in a tube: 0.85, 0.75, 0.65, 0.60, 0.55, 0.50, 0.45, 0.40, 0.35, 0.30, 0.20 and 0.10 per cent sodium chloride. Five ml. of distilled water are placed in an additional tube. Intermediate concentrations of saline such as 0.475 and 0.525 per cent may be helpful for critical work.

3. Add 0.05 ml. of defibrinated blood to each tube.

4. Mix the solutions and allow them to stand at room temperature (20 C.) for 30 minutes.

5. Remix the solutions, centrifuge for 5 minutes at 2,000 R.P.M. and pipet off the supernatant solutions.

6. Hemolysis can be read visually and recorded as the point of beginning and complete hemolysis. However, for more quantitative work, it is desirable to read the supernatant solutions in a photoelectric colorimeter. This is done by making an appropriate dilution (1:2 or 1:5) of the supernatant solutions. The amount of hemoglobin present is determined by the routine method in use in the laboratory. The supernatant solution from the 0.85 per cent sodium chloride tube is used as a blank. The hemoglobin value in the tube containing no saline is taken to be 100 per cent hemolysis and the per cent hemolysis in each of the other tubes is calculated by dividing the hemoglobin value by the value in the tube containing no saline (x 100).

7. The osmotic fragility after incubation of the blood at 37 C. for 24 hours is determined in the same manner as outlined above. However, since the fragility may be markedly increased it is desirable to set up additional saline solutions containing 1.2, 0.90, 0.80 and 0.70 per cent sodium chloride. The 1.2 per cent solution is then used as the blank in the photoelectric estimation of hemoglobin.

8. It is always desirable to run a normal control along with the unknown.

9. The values for per cent hemolysis are plotted on ordinary graph paper on the ordinate and the concentrations of sodium chloride are plotted on the abscissa.

Comments

1. There are many sources for technical errors in this procedure. The saline solutions must be prepared accurately. Experience is

needed to pipet accurately such small volumes of blood. If the pH of the blood-saline mixtures is shifted from pH 7.4 by as much as 0.1 of a pH unit or if the temperature of the solution is altered by 5 C. the tonicity of the solution will be altered by 0.01 per cent. For these reasons, it is always well to run a normal control along with the unknown.

2. Normal values are given in table 50. However, as stated above, it is recommended that each laboratory determine its own values for normal subjects.

Buffered Sodium Chloride Solutions for the Determination of Osmotic Fragility

A reagent grade of sodium chloride should be used. The salt should be dried for 24 hours in a desiccator over calcium chloride.

A stock solution of buffered sodium chloride, osmotically equivalent to 10 per cent sodium chloride is prepared as follows:

Sodium chloride ($NaCl$) 180 Gm.

Dibasic sodium phosphate (Na_2HPO_4) 27.31 Gm.

Monobasic sodium phosphate ($NaH_2PO_4 2H_2O$) 4.86 Gm.

Distilled water to make 2 liters.

This solution will keep for months in a well stoppered bottle.

TABLE 50.—*Normal Values for Osmotic Fragility*

Sodium Chloride Conc. in %	Per Cent Hemolysis: Prior to Incubation	Per Cent Hemolysis: Following Incubation
0.85	0	0
0.75	0	0 — 2
0.65	0	0 — 19
0.60	0	0 — 40
0.55	0	5 — 70
0.50	0 — 5	36 — 88
0.45	0 — 45	54 — 96
0.40	50 — 90	65 — 100
0.35	90 — 99	72 — 100
0.30	97 — 100	80 — 100
0.20	100	91 — 100
0.10	100	100

A 1 per cent working solution is made from the 10 per cent stock solution.

Test solutions equivalent to 0.90, 0.85, 0.80, 0.75, 0.70, 0.65, 0.60, 0.55, 0.50, 0.45, 0.40, 0.35, 0.30, 0.20 and 0.10 per cent sodium chloride are prepared in 50 ml. volumetric flasks. The following volumes of the 1 per cent solution are added by the use of buret to each flask: 45 ml., 42.5, 40.0, 37.5, 35.0, 32 5, 30 0, 27.5, 25.0, 22.5, 20.0, 17.5, 15.0, 10.0 and 5.0. The solutions are then made up to volume with distilled water. A 1.2 per cent sodium chloride solution can be prepared by diluting 6 0 ml. of the 10 per cent solution to 50 ml. These solutions will keep for several weeks at 4 C. but should be discarded if mold develops.

Autohemolysis Test

Technic

1. Two ml. of sterile defibrinated blood are placed into each of four 5 ml. screw-capped vials. The duplicate specimens are set up in the event that one may not be sterile.

2. Serum is obtained from an additional sample of blood and set aside in the refrigerator.

3. The bottles containing the blood are placed in an incubator at 37 C. and left undisturbed. After 24 hours the bottles are gently mixed by inversion and two are replaced at 37 C. and incubated another 24 hours. The other two bottles are inspected for contamination and if none is found the contents are pooled, a sample removed for the estimation of the volume of packed red cells (V P.R.C.), and the remainder centrifuged to obtain the serum.

4. The same procedure is followed for the two bottles removed from the incubator after a total of 48 hours

5 The amount of spontaneous hemolysis is estimated by measuring the amount of hemoglobin in the serum This can be done by diluting the serum 1 in 25 or 1 in 50, depending on the degree of hemolysis Since methemoglobin formation may be considerable under these conditions, the hemoglobin cannot be measured as oxyhemoglobin. The cyanmethemoglobin method (p. 27) is quite satisfactory or the serum may be diluted with 0.1N HCl and the hemoglobin measured as acid hematin. In either procedure a 1 in

100 or 1 in 200 dilution of whole blood is used as a standard and an appropriate dilution of the preincubation serum is used as a blank. The percentage hemolysis, allowing for the change in packed cell volume resulting from incubation, is calculated as follows:

$$\% \text{ Hemolysis} = (D_2 \text{-} D_3) \times \text{Dil. of serum} \quad \frac{100 - \text{V.P.R.C.}}{D_1 \times \text{Dil. of blood}}$$

D_1 = density of diluted whole blood
D_2 = density of diluted serum after incubation
D_3 = density of diluted preincubation serum
V.P.R.C. = volume of packed red cells in ml./100 ml.

Interpretation

1. About 0 to 0.5 per cent hemolysis takes place in normal blood in 24 hours and 0.4 to 3.5 per cent in 48 hours. The rate of hemolysis is accelerated in blood specimens with an increased osmotic fragility.

2. A difference between the rates of hemolysis of normal and abnormal blood can frequently be detected visually. Normally, only small amounts of lysis are visible at the end of 48 hours incubation.

Sickle Cell Test

Technic

1. Place a drop of the blood to be tested on a slide.
2. Place one or two drops of the sodium metabisulfite solution on the slide and mix thoroughly with the drop of blood.
3. Cover with a cover glass and express the excess blood by gently pressing the cover glass with a piece of filter paper.
4. Examine under the high dry objective of the microscope.
5. If the blood is from a patient with sicklemia, sickling will appear immediately and will be marked within 15 minutes. If sickling does not occur within 15 minutes, sicklemia is not present.

Comment

This test may be performed without the addition of the reducing agent by sealing a drop of blood under a cover glass with paraffin

or vaseline. The disadvantage of this simplification is that if one is dealing with the sickle cell trait rather than sickle cell anemia, sickling may not occur for 6 or even 24 hours.

Reagent

Sodium metabisulfite ($Na_2S_2O_5$) solution
Sodium metabisulfite 2 Gm.
Distilled water to make 100 ml.

This solution must be fresh. Tablets containing 0 2 Gm. of sodium metabisulfite may be purchased from A. S. Aloe Company, 5655 Kingsbury, St. Louis 12, Mo. One tablet is dissolved in approximately 10 ml. of distilled water just prior to use.

A buffered solution of sodium dithio*nite* (sodium hydrosulfite, $Na_2S_2O_4$) may be substituted for the sodium metabisulfite ($Na_2S_2O_5$). However, sodium dithio*nate* ($Na_2S_2O_6$) is inactive as a sickling agent.

REFERENCES

1. DALAND, G. A AND CASTLE, W B · Simple and rapid method of demonstrating sickling of red blood cells. J Lab & Clin Med 33 1082, 1948
2. ITANO, H A AND PAULING, L : A rapid diagnostic test for sickle cell anemia Blood 4. 66, 1949

Determination of Fetal Hemoglobin

Technic

1. A solution of hemoglobin containing 9 to 11 Gm. of hemoglobin per 100 ml. is prepared from freshly-drawn clotted, oxalated or citrated blood. A convenient technic is as follows. place 10 ml. of freshly drawn blood in a 15 ml. graduated centrifuge tube containing two drops of a 20 per cent solution of potassium oxalate. Centrifuge at 3000 R.P.M. for 20 minutes Draw off and discard the plasma. Add approximately 10 ml. of physiologic saline to the packed cells. Mix and then centrifuge at 3000 R.P.M for 30 minutes. Draw off and discard the saline Draw off the packed red cells until 2 ml of packed cells remain in the tube. Add distilled water to the 6 ml. mark, followed by 0.8 ml. of toluene (C.P.).

Shake for 5 minutes. The tube is then centrifuged at 3000 R.P.M. for 5 minutes. However, a better recovery of hemoglobin solution is obtained if the mixture is allowed to stand overnight in the refrigerator prior to centrifugation. Discard the upper two layers. Filter the clear red solution. The final solution should contain 10 Gm of hemoglobin per 100 ml. (9 to 11 Gm.) The exact concentration of hemoglobin can be determined (p. 27) and the solution adjusted if necessary.

2. Pipet 1.6 ml of the "alkaline" reagent into a serologic test tube.

3. Place the test tube in a waterbath at 20 C. for 5 minutes.

4 Pipet 0 1 ml. of the hemoglobin solution into the test tube.

5. Rinse the pipet 6 times with the contents of the tube.

6. Shake the tube gently for 10 seconds.

7. Exactly one minute after the hemoglobin is introduced into the "alkaline" reagent, add 3.4 ml. of the "precipitating" solution.

8 Mix by inverting the tube 6 times.

9. Immediately filter the mixture through a double layer of filter paper (Whatman No. 1).

Interpretation

1. The procedure must be followed exactly. The time interval, temperature, pH and concentration of the reactants are critical The final supernatant solution must be filtered and examined immediately.

2 Under the conditions of this test, normal adult hemoglobin (hemoglobin A) is completely denatured (precipitated) within one minute. Therefore, when only normal adult hemoglobin is present, the filtrate will be colorless. If more than two per cent of the hemoglobin is alkali resistant (fetal hemoglobin) the final filtrate will be faintly brown to deeply red in color.

3. The amount of alkali-resistant hemoglobin may be quantitated by determining the amount of hemoglobin in the initial solution and in the final filtrate (p 27). The alkali-resistant hemoglobin is then expressed as a percentage of the initial amount of hemoglobin In normal adult subjects, 0.5 to 1 7 per cent of the hemoglobin is alkali resistant

Reagents

Alkaline reagent

To 10 ml. of exactly 1N NaOH solution add exactly 110 ml. of distilled water. This solution should be kept in the refrigerator in a paraffin-lined bottle. The pH of the solution should be 12.7. If a precipitate forms or if the solution becomes cloudy it must be discarded.

Accurately standardized solutions of one normal sodium hydroxide are prepared commercially (Eimer and Amend, 635 Greenwich Street, New York City) and can be purchased through many sources (for example, Fisher Scientific Company, St. Louis, Mo.). It is not recommended that the student or physician prepare his own standard solutions.

Precipitating solution

Anhydrous ammonium sulfate 37.7 Gm.
Distilled water 100 ml.
Hydrochloric acid (10N) 0.25 ml.

REFERENCE

SINGER, K., CHERNOFF, A. I., AND SINGER, L. Studies on abnormal hemoglobins. I. Their demonstration in sickle cell anemia and other hematologic disorders by means of alkali denaturation. Blood 6: 413, 1951.

Detection of Abnormal Hemoglobins by Paper Electrophoresis

The study of hemoglobin by filter-paper electrophoresis has become a valuable and essential tool in the investigation and diagnosis of hereditary hemoglobin abnormalities. This simple technic does not require the complex and expensive apparatus and special skills necessary for the Tiselius moving-boundary method. A great variety of apparatus and technics, most of which are quite satisfactory, have been described. The apparatus used has varied from the simple, inexpensive, "home-made" variety to expensive, commercial models. The hemoglobin can be made to move as a spot or as an even boundary.

The technical details of the procedure vary with each type of apparatus. *A moderate amount of experimentation by trial and error is necessary with a particular apparatus in order to obtain optimal separation of the hemoglobins. The technic described below* is that of Bergren, Sturgeon and Itano. This particular technic has been selected primarily because the apparatus is simple and inexpensive to construct. The technic is not complicated, the results obtained are quite satisfactory, and seven specimens can be run simultaneously.

The technics described by others are equally satisfactory. References to several of these are given below

Apparatus

The apparatus (fig. 20) consists of two buffer tanks, a bridge to support the paper, four electrodes, and a power supply.

Pyrex "bread baking dishes" which may be purchased at a grocery or hardware store are satisfactory for the buffer tanks. These *should be of such a size as to hold about one liter of solution. As an alternative the buffer tanks can be made out of plastic. A connecting tube between the two buffer tanks facilitates leveling of the buffer.*

The bridge to support the paper consists of a bottom and a top plate Two pieces of window glass, ¼ inch thick and 10 x 12 inches, will suffice.

The electrodes consist of carbon rods. The electrode vessel can be made out of a test tube (20 x 150 *mm.*) (fig 21). *A small hole* is made in the side of the test tube *about one to two cm* up from the bottom *This is accomplished by softening the glass* in a small hot flame, followed by placing a thumb over the open end of the tube The *increasing pressure within the tube* will blow open a small hole *A small pad of glass* wool is then placed in the bottom *of the tube to strengthen the agar bridge.* The tube is placed in a beaker, and 3 *per cent agar* in 2 molar sodium chloride* solution is poured *in to a level of* 3 cm. from the bottom The level of agar should be the same inside and out After the agar has solidified, the tube is removed from the beaker and is filled to near the top

*116.9 grams of anhydrous sodium chloride per liter.

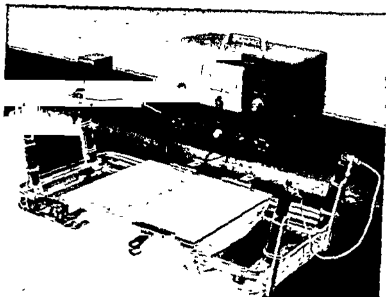


Fig 20.—Showing the paper electrophoresis apparatus described in the text The electrodes are connected to a "Heathkit" variable voltage regulated power supply (Courtesy of Dr W R Bergren)

with a 2 molar sodium chloride solution. The carbon electrode is inserted into the tube. The tip of the electrode should be about one cm. above the level of the agar and is held in place by a rubber stopper at the top. The rubber stopper should have a small hole to allow the gas to escape The electrode vessels are then placed into the buffer in the dish and fastened to the side by means of a tongue depressor and a rubber band. The two electrodes on each end are connected together by means of a wire with a battery clamp on each end.

A simple alternative to the above is to place the carbon electrode in the long arm of a T tube. Glass wool can be placed in the double-outlet ends and no agar is needed.

Power Supply

A power unit capable of supplying up to 500 volts output, with a current flow in the system of up to 200 milliamperes, is desirable.

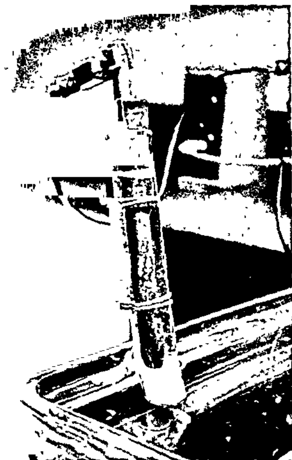


Fig 21.—Showing the electrode vessel and carbon electrode in place. (Courtesy of Dr W R Bergren)

An inexpensive direct current power source may be obtained by removing the power pack from a radio, manufactured when separately mounted units were employed as substitutes for battery operation. The disadvantage of such a unit is that it has a low voltage output and consequently is suitable for use only with buffers of very low ionic strength. A highly satisfactory, relatively

inexpensive unit, which must be assembled by the buyer, can be purchased from the Heath Company, Benton Harbor, Michigan. Many satisfactory preassembled power units are available on the market. However, these are quite expensive.

Preparation of the Hemoglobin Solution

This is described in the section on the determination of fetal hemoglobin (p. 166). The blood samples used for the preparation of hemoglobin solutions must be relatively fresh. They may be stored unfrozen for several days in a refrigerator. Beyond that time they may give marked trailing effect. They may be stored in the frozen state for weeks but should be recentrifuged prior to use.

Preparation for Veronal Buffer (pH 8.6, ionic strength 0.05)

Sodium barbital (Merck, U.S.P.) 10.30 Gm
Barbital (diethylbarbituric acid) 1.84 Gm
Distilled water to make one liter

Approximately one liter is placed in each buffer tank. Care must be taken to put the same amount in each tank. At the low ionic strength employed heating of the filter paper does not occur and the procedure can be carried out at room temperature.

Although the Veronal buffer is used routinely, for the separation of Hb H from Hb I a *phosphate buffer* (pH 7.4) is of value. This is prepared as follows:

Monosodium phosphate, monohydrate 0.6 Gm
Disodium phosphate, anhydrous 2.2 Gm.
Distilled water to make one liter

Preparation of the Paper Bridge Assembly

One of the two glass plates is placed horizontally on a wood block away from the buffer vessels. A sheet of thick filter paper (Schleicher and Schuell No. 598), 9.5 x 11.5 inches, is wet with buffer and carefully centered on the plate. Inclusion of air bubbles must be avoided. A sheet of thinner paper (Whatman No. 1), 9.5 x 16 inches, is wet with buffer and placed over the thicker piece with equal lengths projecting over the plate ends. Blotting is accom-

plished by placing two dry sheets (9.5 x 12 inches) of thick paper (S. and S. 598) over the two wet sheets on the bottom plate. The top plate is put into position and clamps ("bulldog" clips) are applied. Excess buffer on the hanging "tails" is blotted off with scraps of paper. After standing for about 10 minutes, the clamps are removed and the top glass plate is lifted off. If the blotting is not thorough and symmetrical, the hemoglobin spots will be distorted. The two top sheets are discarded and the bridge is now ready for the samples.

Care should be taken to keep the filter paper clean before use and to prevent its exposure to acid or alkaline fumes during storage.

Electrophoresis Run

1. The exact center line on the paper is determined and the hemoglobin solutions are placed on premarked points along the center line. It is essential that the samples be placed on the center line to avoid distortion as the paper fills with buffer. Known samples are alternated with unknown samples. By the use of a capillary tube about two microliters (.001 to .002 ml.) of a hemoglobin solution are placed on the paper. The use of larger volumes results in blurring of the spots. An idea of the size of the capillary to use can be obtained by filling a 0.1 ml. pipet marked in 0.01 ml. divisions with a colored solution.

2. As soon as the samples are on the paper, the top plate is replaced, the clamps are put on, and the assembly is placed in position on the edges of the buffer vessels, with the "tails" hanging freely in the buffer.

3. The power supply is turned on. After the buffer fronts coming from the two vessels have met in the midline (20 to 30 minutes), the voltage is adjusted to about 300 (15 to 18 milliamperes).

4. Migration is considered complete when the leading edges of the normal hemoglobin spots, readily seen through the top plate, have moved about 8 cm. from the origin. The time required is usually about six hours.

Staining of the Hemoglobin Spots

The hemoglobin spots are colored and clearly visible on the

paper. For most purposes, it is sufficient to record in a notebook, the results which are observed. If a permanent record is desired the papers may be dried without prior staining. The paper should be stretched in a horizontal position on a suitable frame, with contact only at the ends. Uneven water evaporation from the paper surfaces results in distortion of the spots. This can be avoided by drying the paper in a draft-free environment.

For the delineation of minor components it is sometimes desirable to stain the paper. This is done by placing the paper (usually the thicker bottom sheet) in a solution of 0.1 per cent bromphenol blue and 10 per cent mercuric chloride in 95 per cent ethanol. After 15 minutes in this solution, the paper is immersed for 5 minutes in 2 per cent acetic acid, followed by washing in water. The blue hemoglobin spots are then visible against a white background. The paper can be dried at room temperature if it is desirable to retain the record.

Comments

1. The unknown abnormal hemoglobin can only be identified by comparing its mobility with the mobility of known abnormal hemoglobins. Therefore, it is necessary to have at least several known abnormal hemoglobins available. Usually specimens containing hemoglobins A, S and C are adequate controls.

2. The optimal voltage, current, and duration of the runs will have to be determined for each apparatus and set of conditions.

3. The pH of the buffer is not critical since relative rather than absolute mobilities are measured.

4. The apparatus detailed above is designed to operate effectively at room temperature. However, if too high a current is employed for a given ionic strength of buffer, excessive heat production will result and the hemoglobin will become denatured. This can be avoided by reducing the potential or, if necessary, using a buffer solution of lower ionic strength.

5. While individual components can be separated into distinct zones, there is usually a small amount of hemoglobin distributed along the pathway between the two components. This is called "trailing." Excessive "trailing" can result from (a) the presence

of stroma in the hemoglobin solution, (b) denaturation of the hemoglobin from ageing or excessive heat, (c) too much hemoglobin placed on the paper, (d) excessive buffer on the paper, and (e) improper or unclean paper.

6. If the volume of the sample applied to the paper is too great or the hemoglobin concentration of the sample is too high, or if too many samples are applied to the sheet, the spots will not migrate in straight lines but will crowd out toward the edges of the paper.

Interpretation

In Veronal buffer, pH 8.6, all of the hemoglobins carry negative charges and migrate toward the positive electrode (figs. 22 and 23). Hemoglobin H and I have the highest mobility. The others follow in the order given: $H = I > J > K > A > F > G > S = D > E > C$. However, only under the most optimal conditions can the components be clearly separated from one another. For the detection of minor components, or for the separation of mixtures of fractions with closely similar mobility, the method is often not sufficiently discriminating.

The mobilities of Hb H and Hb I are very similar and it is usually impossible to distinguish them at pH 8.6 (fig. 23). However, at pH 7.1 (phosphate buffer) the mobility of Hb H is sufficiently greater than the mobility of Hb I to allow identification (fig. 23).

The presence of an increased amount of Hb F often causes a "drag," i.e., a slowing down of other components. Thus, it is frequently impossible to separate Hb F from Hb A, although Hb F is clearly the slower component of the two (fig. 22, left). Frequently, Hb F appears as a "tail" on the Hb A spot. The presence of an increased amount of Hb F can be detected by the alkaline denaturation test (p. 166). Hb F cannot be readily distinguished from Hb G on paper (fig. 22, left). The separation of Hb F from Hb S may also be unsatisfactory. However, Hb S can be distinguished from Hb F by the sickle cell test (p. 165).

Hb G in combination with Hb S may be difficult to detect. Again the sickle cell test will distinguish the two hemoglobins.

The electrophoretic mobility of Hb S is identical with the mobility of Hb D at pH 8.6 (fig. 22, right).

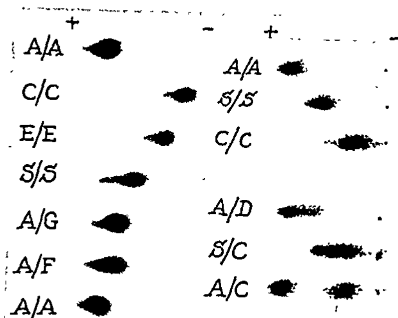


Fig 22.—Paper electrophoretic patterns Veronal buffer pH 8.6 Pattern on the right was kindly furnished by Dr W R. Bergren (Courtesy Acta Haemat) The pattern on the left was kindly furnished by Dr A I Chernoff (Courtesy New England J Med)

The electrophoretic mobilities of Hb A and Hb C, Hb A and Hb S, Hb A and Hb D, and Hb S and Hb C are sufficiently different to make identification easy (fig. 22).

REFERENCES

1 Bergren, W R, Sturgeon, P, and Itano, H Zone electrophoresis of abnormal hemoglobins Separation on paper of hemoglobins associated with sickle cell disease Acta Haemat *12* 160, 1954

2. Motulsky, A G, Paul, M H, and Durrum, E L Paper electrophoresis of abnormal hemoglobins and its clinical applications A simple semiquantitative method for the study of the hereditary hemoglobinopathies Blood *9* 897, 1954

3 Smith, E W and Conley, C L Filter paper electrophoresis of human hemoglobins with special reference to the incidence and clinical significance of hemoglobin C Bull Johns Hopkins Hospital *93* 94, 1953

4 Itano, H A, Bergren W R, and Sturgeon, P The abnormal human hemoglobins Medicine *35* 121, 1956

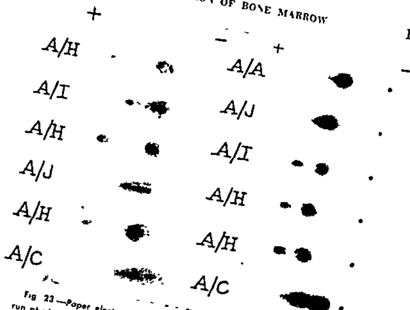


Fig 23.—Paper electrophoretic patterns The pattern on the right was run at pH 8 6, Veronal buffer, ionic strength 0 05 Under these conditions Hb H cannot be distinguished from Hb I The pattern on the left was run at pH 7 4, phosphate buffer, ionic strength 0 032 Under these conditions Hb H moves faster than HB I Patterns are by courtesy of Dr W R Bergren

Examination of Bone Marrow

Two different types of technic are available for the study of marrow These two methods give information which is supplementary rather than complimentary (1) a small amount of bone marrow may be obtained by needle aspiration, and from this material a thin smear can be prepared and stained with Wright's stain, (2) fixed thick sections of bone marrow stained with hematoxylin and eosin may be prepared from material obtained from needle aspiration or from material obtained by surgical biopsy of the marrow

In thin smears stained with Wright's stain individual cells can be identified and fine morphologic details can be observed. In

thick sections identification of individual cell types is not possible. On the other hand, the histologic pattern and cellularity of the marrow can be observed in thick sections but not in thin smears. Judging the cellularity of the marrow from thin smears may be misleading.

The disadvantages of surgical biopsy of the bone marrow are that it is a more complicated procedure than needle aspiration from the standpoint of convenience to the patient, danger involved, expense and time, and it involves the coordinated efforts of the internist, surgeon and pathologist. Preparations of thick sections from aspirated material have the advantages over surgical biopsy because they are more convenient, less expensive, and less time consuming for the patient. However, more skill is involved in obtaining satisfactory preparations.

Cytologic study of cells aspirated from bone marrow may be of great value in the diagnosis of hematologic disease and in the understanding of the pathologic physiology of the hemopoietic tissues. Because of this, it is fortunate that the marrow cavity of many bones is easily accessible with a minimal amount of discomfort or danger to the patient, and that marrow can be obtained with only moderate experience and skill on the part of the operator. Unfortunately, the procedure is of limited value and will not solve all types of hematologic problems Therefore, it is important to have clearly in mind the situations where marrow examination can be expected to yield crucial information which cannot be extracted from the history, physical examination or examination of the blood.

Indications, Limitations, Contraindications and Complications

Examination of thin smears of bone marrow is most helpful in those conditions in which diagnostic cells are observed in the bone marrow but are not usually present in the blood. These conditions are (1) multiple myeloma, (2) tumor cells metastasizing from prostate, breast, lung, kidney and other tissues, (3) Gaucher's disease, (4) Niemann-Pick disease, (5) kala-azar, (6) histoplasmosis, and (7) megaloblastic anemia

In most patients with pernicious anemia the diagnosis can be

made from the history, physical examination and blood. Rarely is it necessary to resort to marrow examination to make the diagnosis of this disease. This statement does not apply, however, to the megaloblastic anemias which occur in infancy and during pregnancy. In these conditions, the alterations in the blood may not be as striking as they are in pernicious anemia. Indeed, the anemia may be normocytic even though the bone marrow is megaloblastic.

A second situation in which examination of thin smears is frequently helpful is in the group of conditions characterized by pancytopenia (table 19). If the pancytopenia is the result of leukemia in the "aleukemic" form, marrow examination will permit an unequivocal diagnosis to be made. Marrow examination is particularly helpful in ruling out the diagnosis of leukemia in those patients with pancytopenia from some other cause.

Examination of bone marrow smears is of little or no diagnostic value in microcytic hypochromic anemia, posthemorrhagic anemia, hemolytic anemia, hemorrhagic disorders, lymphoma, anemia of nephritis, anemia of infection, polycythemia, myelosclerosis, infectious mononucleosis, chronic myelocytic leukemia, or in chronic lymphocytic leukemia

Paraffin sections made from particles of bone marrow removed by aspiration are particularly of value in patients with undiagnosed hematologic disease in disclosing the presence of granulomatous lesions. Such lesions have been found in the bone marrow of patients with tuberculosis, sarcoidosis, brucellosis, histoplasmosis, infectious mononucleosis and malignant lymphoma. Sections are also of value (1) in detecting the invasion of marrow by myeloma cells, carcinoma cells and Gaucher cells and (2) in studying the cellularity and histologic architecture of the marrow The cellularity of the marrow is of particular interest in the diagnosis of aplastic anemia and the histologic pattern is of interest in the diagnosis of myelofibrosis.

The value of bone marrow examination is limited in diseases which involve the marrow in a patchy fashion since the lesion may be completely missed. This statement applies to both thin smears and thick sections, but, of course, more to the former than to the latter. In some patients, no marrow at all will be obtained after repeated attempts at various sites. Furthermore, "dry taps" are more common in patients with granulomatous lesions, myelofibrosis,

and metastatic carcinoma than in normal subjects or patients with myeloid or erythroid hyperplasia. This is because these lesions are frequently associated with some fibrosis of the marrow and the cells remain fixed in the marrow cavity. Surgical biopsy of the marrow is indicated in those patients in whom such lesions are suspected and in whom one or more "dry taps" have been obtained.

The only contraindication to marrow aspiration is in hemophilia and related disorders.

Although marrow examination potentially may permit infection or serious hemorrhage, these two complications rarely occur when proper technic is used. Excessive bleeding sometimes occurs in patients with thrombocytopenia but it can usually be satisfactorily controlled by pressure over the site of the puncture. The only serious complication of marrow aspiration is cardiac tamponade when the lower plate of the sternum is perforated as a result of inexperience or carelessness.

Technic

Site of Puncture. Various sites may be used for the puncture. *The sternum is very satisfactory and is the most commonly used* site in adults. Marrow will be found at this site even in infants of one to two years of age. The chief disadvantage in using the sternum is that the patient can observe the procedure and may have considerable apprehension due to the proximity of the sternum to the heart. In children the sternal site is less satisfactory than other sites because of the difficulty in completely immobilizing the chest during the procedure, and because the sternal-marrow cavity is quite shallow and the possibility of perforation into the mediastinum, although remote, is always present. When the sternum is used, the upper portion of the bone, between the second and third ribs in the midline, is most suitable, and the needle is inserted perpendicular to the bone with the guard set at a depth of 1 cm for adults or at 0.2 to 0.6 cm. for a child. *Iliac crest* puncture is extremely satisfactory in infants and children and in apprehensive adults and has the advantage that no vital centers are near the site of puncture. *The patient is placed on his back or side and the* bone marrow needle is inserted into the ilium from the lateral

approach about 2 cm. below the anterior superior spine. *Spinous process* puncture may be performed with the patient in the sitting position or lying face downward. The lumbar and lower thoracic vertebrae are the most satisfactory. With the guard set at 1.5 to 2.0 cm. the needle is inserted directly into the end of the spinous process, midway between the upper and lower border. Occasionally the needle must be advanced a few mm. at a time to secure marrow. An alternate procedure is to introduce the needle just below the tip of the process on one side; then the needle is directed medially. In children from birth to 4 to 5 years of age, the safest bone to puncture is the *tibia*. This site also has the advantage of being easily immobilized The needle with the guard set at about 2 cm. is inserted just below the tubercle on the medial or anterior aspect. The bone may be quite hard in the older infant and considerable pressure may be necessary to penetrate into the marrow cavity.

Aspiration. The procedure should be discussed with either the patient or the parent or both in order to prevent undue apprehension If after this the patient is still apprehensive, it is well to administer a sedative a half-hour before the procedure. The operator should scrub as for any surgical procedure Sterile gloves are desirable but not necessary The skin is shaved if necessary, washed, and cleaned with iodine and alcohol or other suitable antiseptics. The area may be draped with sterile towels, although if care is taken, this is unnecessary The skin, subcutaneous tissues, and finally the periosteum of the bone are infiltrated with procaine After a suitable time interval the adjustable guard on the special marrow needle* is set at a suitable depth and the needle is inserted into the bone with a rotating or boring motion A "give" is felt when the marrow cavity is entered The needle is then passed 1 or 2 mm into the cavity. The stylet is removed from the needle and a dry, sterile, tight fitting 5 ml syringe is attached Firm but gentle pressure is applied to the plunger of the syringe until the first drop of marrow appears If the needle is in the marrow cavity, momentary pain is usually experienced by the patient when suction is applied. If no marrow appears, it is necessary to replace the stylet and move

*A satisfactory needle, called the University of Illinois Sternal needle, is manufactured by V Mueller and Company, 408 South Honore Street, Chicago 12, Ill

the needle. As soon as one drop of marrow appears in the syringe, the syringe is removed and passed to an assistant who then prepares several smears. If desired, a second dry sterile syringe is then attached and 1.0 ml. of marrow is withdrawn. After removal of the marrow needle, a small, tight, sterile dressing is applied.

Preparation of Thin Smears. From the several drops of marrow in the first syringe, thin smears are prepared and stained with Wright's stain, except that the fixing time and the staining time are increased to about twice that required for blood. Bone marrow smears may also be stained by the peroxidase method (p. 91), alkaline phosphatase method (p. 93) or Prussian blue method (p. 87).

Preparation of Thin Smears From Concentrated Marrow. The marrow obtained in the second syringe is placed in a vial containing a small amount of either Versenate or heparin. After proper mixing, a hematocrit tube is filled and centrifuged for 5 minutes at 1000 R.P.M. Four layers may then be distinguished: fat, plasma, nucleated cells and erythrocytes. Measurement of the volume of these layers is of limited value and may be misleading, but the fat and plasma can be pipetted off and smears made from the nucleated cell layer diluted with an equal volume of plasma. In this manner concentrated material can be obtained from the nucleated cell layer. This is of particular value when the direct smears are relatively acellular or when a particular abnormal cell type is present in small numbers. The marrow need not be concentrated routinely

Examination of Thin Smears. All preparations should be examined first under low power. Certain cells such as megakaryocytes, Gaucher cells and carcinoma cells may be few in number and not evenly dispersed throughout the smear. By virtue of their size they may be picked out easily under low power. They may then be examined under higher magnification for positive identification If the examination is restricted to the oil-immersion objective they may not be seen at all. It is not uncommon for carcinoma cells to cluster in one small area of the cover glass.

After the entire area of the cover glass has been examined with the low power objective, a good area for detailed examination under the oil-immersion objective is selected. The cells in this area should be almost but not quite touching one another. Cells cannot be

identified in an area which is too thick. In the areas which are too thin, the morphology of the cells may be disturbed. Selection of the appropriate area to make the detailed examination is one of the most important steps in the examination of marrow. This should be done with great care.

In certain circumstances it may be desirable to perform a differential cell count. This is done on a total of 300 to 500 cells and the results for each cell type are recorded as a per cent of total cells counted. The normal mean and range for each cell type is given in table 51.

Calculation of the myeloid: erythroid (M:E) ratio may be helpful (table 52). This is done by dividing the total number of myeloid cells (myeloblasts, promyelocytes, myelocytes, metamyelocytes and PMN's) counted by the total number of erythroid (pronormoblasts and normoblasts) cells counted. If information concerning only the M:E ratio is desired, the procedure may be shortened by grouping all of the myeloid cells into one group and the erythroid cells into a second group. The nonmyeloid cells may be ignored and the particular stage of maturation of the myeloid and erythroid cells may be ignored.

In certain instances it is instructive to plot a "maturation" curve for the myeloid series of cells. This is done by adding up the total number of cells counted in the series and calculating the percentage of each cell type within the series. The percentages are then plotted as illustrated in figure 24.

A "maturation" curve for the erythroid series of cells may also be obtained (fig. 25).

Bone Marrow Hemosiderin. Thin smears of bone marrow stained with Wright's stain may be counterstained with Prussian blue (p. 87) for the presence of stainable iron in the erythroid series. However, hemosiderin granules are not found readily in thin smears. Particles of marrow must be examined. A convenient manner to obtain marrow particles for this purpose is to draw about 3 ml. of a solution of 1 per cent sodium citrate into a syringe. About 3 ml of mixed blood and marrow are drawn into the syringe. The mixture is placed on a large watch glass, particles of marrow are identified, teased out, and smeared on cover glasses.

The unstained preparation is examined first under low power magnification with reduced illumination in order to locate an area

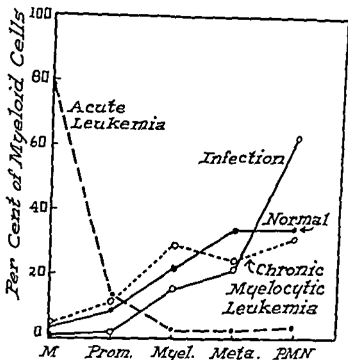


Fig 24.—Representative myeloid "maturation" curves M = myeloblast, Prom. = promyelocyte, myel = myelocyte, meta = metamyelocyte, PMN = polymorphonuclear

of marrow tissue. After a suitable area has been located, it is examined under oil-immersion magnification The hemosiderin appears as golden-yellow, refractile granules, varying from a fraction to several micra in diameter.

For the inexperienced observer, the marrow hemosiderin may be more easily identified after staining with Prussian blue The stain for this purpose is prepared by adding concentrated hydrochloric acid to 20 ml. of a 4 per cent solution of potassium ferrocyanide in distilled water until a white precipitate is formed The solution is then filtered and the smears covered with the filtrate for 30 minutes and then examined as detailed above.

In the stained preparation the granules are blue in color. Not infrequently, structures other than hemosiderin will stain, usually in proportion to the amount of iron present. These artifacts can be

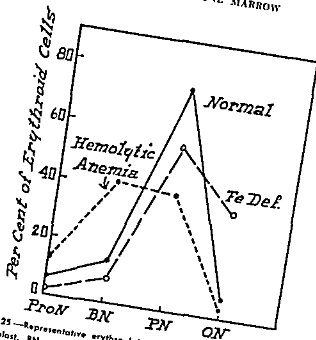


Fig 25—Representative erythroid "maturation" curves Pron = pronormoblast, BN = basophilic normoblast, PN = polychromatophilic normoblast, ON = orthochromic normoblast

recognized with experience In iron deficiency, pale yellow granules which do not take the iron stain are sometimes seen These are probably protein granules of hemosiderin which contain absorbed bilirubin but not iron Normal marrow usually contains a few small granules distributed throughout The number of granules is reduced in patients with reduced iron stores (iron deficiency) and increased in patients with large stores of iron (infection, cirrhosis, hemochromatosis, pernicious anemia, malignancy, uremia and hemolytic anemia)

Preparation of Sections If a thick section is to be prepared, about 1 ml of marrow is drawn into the second syringe This is then placed in a small paraffin-coated vial containing a small quantity of powdered or liquid heparin and mixed by gently rotating The contents of the vial are then poured into a Petri dish Grossly visible particles are usually present Visualization of the particles

may be aided by placing a bright light underneath the dish. The excess blood is drawn off with care so as not to disturb the particles; the particles are teased out with a needle and placed in a small vial containing Zenker's fluid. The marrow particles are fixed for 30 minutes to one hour, depending upon the size of the particles. The fluid, but not the particles, is removed from the vial by means of a capillary pipet.

The particles are then washed with (1) several changes of distilled water (30 min.), (2) 30 per cent alcohol (30 min.), (3) 50 per cent alcohol (one hour or longer), (4) 70 per cent alcohol (one hour or longer), and (5) 95 per cent alcohol (5 min.). Add 100 per cent alcohol and after 5 minutes add an equal volume of xylol. Then wash twice with xylol for 10 minutes each time. Remove the xylol, pour paraffin (m.p. 54 C.) into the vial and allow the tissue to become infiltrated in an oven for 30 to 45 minutes. Remove the paraffin with a warm capillary pipet, add fresh paraffin, and leave the bottle in the oven for not more than 45 minutes. Remove the particles from the paraffin by means of a heated capillary pipet. Place the tip of the pipet at the bottom of a paraffin-filled boat and force the particles out of the pipet. The particles should be made to aggregate in a relatively compact mass near the bottom of the boat. The paraffin is allowed to harden and sections 5 micra thick are cut. The sections are then stained with hematoxylin and eosin or any other stain desired. The details of the hematoxylin and eosin staining procedure are not given here since they are available routinely in all departments of pathology. Certain modifications have been recommended for marrow particles. Several of these are listed in the references.

Interpretation of Thin Smears

The cellular composition of normal bone marrow in adults is given in table 51. The normal myeloid-erythroid ratio for adults is 2 to 5:1 The pattern in infants and children is somewhat different from this and varies according to age. The myeloid erythroid ratio is low at birth (1.8:1), increases rapidly during the first two weeks to values as high as 11:1 and then gradually decreases to 3 1 during the first year (fig. 26). Lymphocytes are low at birth as compared with normal adult values. During the first weeks of life, the lymphocytes increase in number to values as high as 10 per cent. They

TABLE 51—*Normal Bone Marrow*

Cell Type	Mean	Range
Myeloblasts	2.0	0.3—5.0
Promyelocytes	5.0	1.0—8.0
Myelocytes, Neut	12.0	5.0—19.0
Eos	1.5	0.5—3.0
Bas.	0.3	0.0—0.5
Metamyelocytes	22.0	13.0—32.0
P.M.N., Neut	20.0	7.0—30.0
Eos	2.0	0.5—4.0
Bas.	0.2	0.0—0.7
Lymphocytes	10.0	3.0—17.0
Plasma Cells	0.4	0.0—2.0
Monocytes	2.0	0.5—5.0
Reticulum Cells	0.2	0.1—2.0
Mitotic Cells	0.0	0.0—2.0
Megakaryocytes	0.4	0.0—3.0
Pronormoblasts	4.0	1.0—8.0
Normoblasts	18.0	7.0—32.0
Megaloblasts	0.0	0.0—0.0
Myeloid Erythroid Ratio	3.1	2.1—5.1

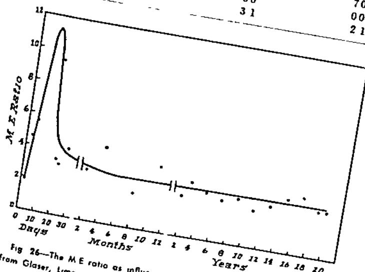


Fig 26—The M E ratio as influenced by age. The data were obtained from Glaser, Limarzi and Poncher (Pediatrics 6 789, 1950)

then gradually decrease during the first and second years, thereafter reaching the adult values

TABLE 52.—*A Summary of Various Types of Reactions Which May Be Observed in Thin Smears from Material Aspirated from the Bone Marrow*

I. Presence of Cells Which Are Not Normally Found in Marrow
  A. Myeloma cells
  B. Carcinoma cells
  C. Gaucher cells
  D. Niemann-Pick cells

II. *Presence of Parasites*
  A. Leishmania
  B. Histoplasma
  C. Malaria

III. M.E Ratio Normal (2–5:1) or *Variable*
  A. Normal marrow
  B. *Aplastic anemia*
  C. Myelosclerosis
  D. Myelophthisic anemia

IV. M.E Ratio Decreased (0.5—2:1)
  A. Due to Increase in Erythroid Cells
    1. Normoblastic hyperplasia
      a. Posthemorrhagic anemia
      b. Iron deficiency anemia
      c. Hemolytic anemia
      d. Cirrhosis of liver
      e. Polycythemia vera
      f. Plumbism
    2. Megaloblastic hyperplasia (see table 15)
  B. Due to Decrease in Myeloid Cells
    1. Agranulocytosis

V. M.E Ratio Increased (5+:1)
  A. Due to Increase in Myeloid cells
    1. Acute myeloblastic leukemia
    2. Chronic myelocytic leukemia
    3. *Infections*
    4. Leukemoid reactions
  B. Due to decrease in Erythroid Cells (rare)
    1. Hypoplastic anemia of infancy
    2. Erythroid hypoplastic anemia in adults

VI. Increase in Nonmyeloid Cells
  A. Acute lymphoblastic leukemia
  B. Chronic lymphocytic leukemia
  C. Aplastic anemia
  D. Infectious mononucleosis

The various types of bone marrow reaction in disease are classified in table 52. This classification is incomplete but it covers the sual type of reaction in the conditions listed. A decrease in the :E ratio due to erythroid hyperplasia, as a general rule, is associated with an increased rate of erythropoiesis. Therefore, in any condition accompanied by hemolytic disease the M:E ratio may be reduced.

In the section which follows, an attempt will be made to give a brief summary of the most important and most characteristic changes which occur in a number of diseases which involve the hemopoietic system.

Multiple myeloma. The characteristic finding is the "myeloma cell." The remainder of the cellular constituents are not remarkable. The myeloma cells seen in different cases range from very immature, anaplastic forms to very mature cells which cannot be distinguished from the adult plasma cells seen in normal marrow. The percentage of myeloma cells found ranges from 3 to 96. It has been suggested that the more immature the cells, the poorer is the prognosis.

The immature myeloma cell is large (15 to 30 μ), round or ovoid, and contains a round nucleus about 5 to 7μ in diameter. In the immature cell the nucleus is frequently but not always eccentrically placed. The nucleus usually contains one, or several, very prominent, deep blue nucleoli. The chromatin pattern is fine and recticular. The cytoplasm is light blue in color and is devoid of granules. Needle-shaped azurophilic inclusion bodies may be present in the cytoplasm.

The more mature myeloma cells are smaller in size (7 to 12 μ) than the immature variety. The mature cell is usually "egg-shaped," that is, narrower on one end than on the other. The nucleus is eccentrically placed. The nuclear chromatin is coarse and dense and occasionally has the so-called "cartwheel" arrangement. Nucleoli are absent. The cytoplasm is deep blue in color, but it does not stain uniformly. There is usually a "cytoplasmic clear area" on one side of the nucleus. The cytoplasm frequently contains small vacuoles, particularly about the periphery. Granules are absent.

Gaucher's Disease. The diagnosis of this disease by marrow aspiration is dependent upon the identification of the very dis-

tinctive Gaucher cells. These cells are large (20 to 80 μ in diameter) and are most easily located under low power magnification. *The most characteristic feature of the Gaucher cell is the abundant,* pale-staining cytoplasm. The cytoplasmic stain may be so pale that under low power it may not be visualized The nuclei may appear to be barren of cytoplasm and to rest in an "open space" some distance from other cells. Fibrillae in the cytoplasm may be observed under oil-immersion. One or many nuclei may be present in a cell. Nucleoli are usually not seen. The chromatin pattern is frequently fine and may resemble that of a reticulum cell.

In most, but not all patients with Gaucher's disease, the typical cells can be found in the marrow. The number present on a single cover glass may vary from several to many.

Niemann-Pick's Disease. The cells of Niemann-Pick's disease are large, the average diameter being about 40 mμ. The most characteristic feature of these cells are the many small hyaline droplets which occur in the cytoplasm and give to the cytoplasm a "honeycomb" or "foamy" appearance. *These droplets stain with Sudan* III and other fat stains. The cells contain one or two nuclei, rarely more.

Aplastic anemia. Material obtained from aspirated marrow frequently consists chiefly of adult erythrocytes However, the marrow is rarely totally acellular in this condition and a "nest" of marrow cells may be aspirated. The differential count is variable and the M:E ratio may be normal or greatly increased or decreased, and consequently, is of little diagnostic value. The most characteristic feature of the marrow is a relative increase in lymphocytes, monocytes and plasma cells. Megakaryocytes are decreased. *There are no features diagnostic of the disease. Thick sections are of greater value* than thin smears because the histologic architecture may be studied

Myelosclerosis. Marrow aspiration is of little value in the diagnosis of this condition. The outer plate of the sternum is penetrated *with difficulty and frequently little or no material is obtained.* The fibroblasts are fixed firmly in the marrow cavity and are not seen in thin smears. The differential count is variable. *If there is* a myeloid reaction in the blood, the M:E ratio *may be increased.* On the other hand, *if a hemolytic process is present, the M:E ratio* may be decreased. Surgical biopsy of the bone marrow is required

to confirm the diagnosis. Stained sections of bone marrow reveal diffuse fibrosis.

Posthemorrhagic Anemia. The normoblasts are increased in number with the result that the M:E ratio is reduced. The degree of reduction of the ratio depends upon the degree of blood loss, the rate of blood loss and the interval of time between the blood loss and the marrow study. Polychromatophilic normoblasts predominate with little or no shift in the "maturation" curve.

Iron Deficiency. The bone marrow is hyperplastic and there is a relative as well as an absolute increase in normoblasts. The increase in normoblasts is roughly proportional to the degree of anemia. Polychromatophilic normoblasts predominate, but there is a tendency for the "maturation" curve to be shifted slightly to the right (fig. 25). Bone marrow hemosiderin and siderocytes are virtually absent. The adult red cells are pale and small.

Hemolytic Anemia. There is a relative as well as an absolute increase in the normoblasts. The increase in normoblasts is roughly proportional to the severity of the hemolytic process. Polychromatophilic normoblasts usually predominate, but in those patients with an extremely rapid rate of hemolysis, and consequently a very rapid rate of regeneration, basophilic normoblasts may predominate (fig. 25).

Cirrhosis of the Liver. Anemia is present in 55 to 90 per cent of patients with chronic liver disease. Anemia may occur as a consequence of (a) an increase in plasma volume, (b) acute or chronic blood loss, (c) a dietary deficiency of folic acid, or (d) impaired liver function per se.

In patients with chronic liver disease and no reduction in the total circulating red cell mass, the morphology of the red cells is normal and the bone marrow is within normal limits. In those patients with anemia secondary to acute or chronic blood loss, the blood and bone marrow are the same as described above for acute posthemorrhagic anemia or iron deficiency. In the few patients with anemia secondary to a dietary deficiency of folic acid, the bone marrow contains typical megaloblasts.

The most frequently encountered type of anemia observed in patients with liver disease is macrocytic. This type of anemia is observed in the absence of such complications as blood loss, folic

acid deficiency and infection, and is considered to be due to the liver disease per se. The anemia is usually accompanied by a slight reticulocytosis and recent studies have provided evidence that an extracorpuscular hemolytic process is present which is proportional in severity to the degree of anemia. The number of normoblasts in the marrow is increased. Large polychromatophilic normoblasts (macronormoblasts) predominate. In addition, it is not uncommon to observe an increase in the proportion of normal mature plasma cells to levels of 3 to 25 per cent. In general, the degree of plasmacytosis correlates directly with the degree of hyperglobulinemia present in the blood.

Polycythemia Vera. The marrow is hypercellular. The hyperplasia involves all marrow elements so that the differential cell count may not be strikingly different from the normal. Frequently, the percentage of normoblasts is increased and there may be a slight shift in the myeloid series of cells to the immature forms. The number of eosinophilic and basophilic leukocytes may be increased. Megakaryocytes are frequently increased

Plumbism. The bone marrow is cellular with an increase in the proportion of normoblasts. The more mature forms predominate. Stippled normoblasts are prevalent.

Anemia of Nephritis The bone marrow is cellular. The M:E ratio is not significantly altered from the normal range of 2 to 5:1. No abnormal cells are present. There are no significant diagnostic changes.

Megaloblastic Anemia. The common denominator among all of the macrocytic megaloblastic anemias (table 15, p 19) is the presence of megaloblasts in the bone marrow. In addition, there are morphologic alterations in the myeloid cells which are of aid in identifying this group of diseases.

The term megaloblast was used by Ehrlich to refer to the type of erythroblast seen in pernicious anemia in relapse This cell is seen only in pernicious anemia and in the other related megaloblastic anemias (table 15) Stages in the development of this cell may be observed which are similar to those found in the normoblast series, namely, promegaloblast, basophilic megaloblast, polychromatophilic megaloblast, and orthochromic megaloblast. There are three features which distinguish normoblasts and the megaloblast series of cells The most important difference is in the nature of

the nuclear chromatin. In the megaloblast series of cells the nuclear chromatin is fine and there is little or no tendency for it to clump as the cell series matures The nucleus may have a sievelike appearance with round areas of parachromatin between the chromatin strands. The chromatin strands may have a beadlike appearance. The nuclear chromatin of the pronormoblast is, of course, relatively fine but as the normoblast series matures the chromatin condenses so that in the orthochromic normoblast the nucleus is nearly solid with chromatin with few or no areas of parachromatin. For this reason, it is necessary to compare the chromatin structure of the pronormoblast with the promegaloblast, the basophilic normoblast with the basophilic megaloblast, and so forth. It is usually easier for the neophyte, or for that matter the expert, to distinguish the orthochromic megaloblast from the orthochromic normoblast than it is to distinguish the pronormoblast from the promegaloblast. The second most important characteristic which distinguishes the two-cell series is the tendency for early hemoglobinization in the megaloblast series of cells Thus, cells will be observed which are the size of pronormoblasts, or larger, which have a nucleus with fine chromatin, but the cytoplasm will be late polychromatophilic in its staining characteristics. Finally, megaloblasts are larger than normoblasts at comparable stages of development. However, this is not the most reliable distinguishing feature.

In addition to the morphologic changes in the erythroid cells, the myeloid leukocytes are strikingly altered. Extraordinarily large leukocytes will be found. These large cells may be present at any stage in the development of the myeloid series but are particularly prominent among the metamyelocytes. Giant metamyelocytes with a large U-shaped nucleus are seen not uncommonly. Large polymorphonuclear neutrophils possessing nuclei with 6 to 10 lobes are likewise observed.

It must be admitted that for the inexperienced the differentiation of megaloblasts from normoblasts may be difficult The most common error is to interpret a marrow containing an increase in pronormoblasts as megaloblastic This error can be avoided by placing greater emphasis on the nature of the nuclear chromatin than on the size of the cells, by examining the later stages of development of the cells in addition to the earlier stages, and by looking for the presence or absence of morphologic alterations in

the leukocytes. Consideration should be given to all of these features before arriving at a conclusion. Admittedly, a single, very immature pronormoblast may not be distinguishable from a promegaloblast even by an expert. However, the expert never allows his opinion to be based on the appearance of a single cell.

Agranulocytosis. In this condition the granulocytic series of cells is reduced with the result that the M:E ratio is reduced. The type of myeloid cell which predominates depends on the duration of action and potency of the offending agent. The first change which occurs is a reduction in the number of mature neutrophils with a shift in the "maturation" curve to the more immature stages. As the agent continues to act the metamyelocytes gradually disappear, then the myelocytes and finally the promyelocytes. Thus, the maturation curve obtained varies with the stage and severity of the disease. When the offending agent is removed, the marrow returns to normal in stages with a progressive shift of the "maturation" curve to the right, provided the alterations are reversible.

Infections. In patients with infection and leukocytosis in the blood, the M:E ratio is greatly increased. The predominate myeloid cell is the neutrophil, and the "maturation" curve is shifted slightly to the right, if it is influenced at all (fig 24).

Acute Leukemia. The bone marrow in this condition is characterized by a great increase in blast forms As a result the myeloid "maturation" curve is shifted far to the left (fig 24) and the M:E ratio is greatly elevated.

In the usual case the preparation is very cellular, and under low power magnification there is a uniformity of cell type giving a monotonous appearance to the smear. The blast forms usually constitute 50 per cent or more of all of the cells in the marrow. This is true regardless of the number of blasts in the blood. This is to say, the marrow is just as "leukemic" in patients with "aleukemic" acute leukemia as it is in patients with a marked leukocytosis and a high proportion of blasts in the blood. Under oil-immersion the same uniformity of cell type is observed The blast forms are identified primarily by the immature structure of the nucleus. In some patients, particularly those with lymphoblastic leukemia, nucleoli may not be prominent and the presence of observable nucleoli cannot be relied upon as a diagnostic feature of the blasts.

In about 90 per cent of patients with acute leukemia the blast cells constitute 50 per cent or more of the total number of cells examined. In 10 per cent of the patients the percentage of blasts will be less than 50. Rarely, patients will be observed in the "pre-leukemic" stage and there may be no increase in the proportion of blasts.

In some patients the differentiation between myeloblasts, lymphoblasts and monoblasts may be made easily because there will be sufficient maturation within the series to allow identification of promyelocytes, lymphocytes or monocytes. In the absence of such maturity the identification of the type of blast may be extremely difficult even for the experienced morphologist. Medical students cannot be expected to become proficient in this matter. The features which distinguish these three types of blasts are outlined in table 53.

Although lymphoblasts may be observed which are as large as

TABLE 53.—*Distinguishing Features of Myeloblasts, Lymphoblasts and Monoblasts*

Characteristic	Myeloblast	Lymphoblast	Monoblast
Cell Size, µ	10–18	10–18	12–20
Cell Border	Even	Even	Irregular Pseudopods
Shape of Nucleus	Oval	Oval	Indented Lobular
Nuclear Membrane	Fine, Smooth	Coarse	Fine, Delicate
Nuclear Chromatin	Fine, Even Delicate, Little Condensation	Moderately Coarse with Some Condensation	Fine, Lacy, No Condensation
Nucleoli	2–5	1–2	2–5
Cytoplasm	Blue No Granules	Blue No Granules	Grayish blue Granules
Differentiation	Promyelocytes	Lymphocytes	Monocytes
Peroxidase	+	—	±

some myeloblasts and monoblasts, in general, lymphoblasts tend to be smaller than other forms of blast cells. The cell border of myeloblasts and lymphoblasts is usually even and smooth. The cell outline of monoblasts is commonly irregular or serrated and pseudopodia may be present. The nucleus of myeloblasts and lymphoblasts is usually oval and so placed in the cell that there is a wider area of cytoplasm on one side of the cell. The nucleus of the monoblast

is usually placed centrally and is frequently indented, kidney-shaped or even lobular. The nuclear membrane of the myeloblast and monoblast is smooth, even and fine. In contrast, the nuclear membrane of the lymphoblast is coarse. The nuclear chromatin of the monoblast is fine and reticular, of the myeloblast even and diffuse, with little condensation. On the other hand, the nuclear chromatin of the lymphoblast is moderately coarse with some aggregation and condensation of the strands, particularly near the inner surface of the membrane. *Nucleoli tend to be more numerous and more conspicuous* in myeloblasts than in lymphoblasts The nucleoli may or may not be prominent in monoblasts. The cytoplasm of the *monoblast is grayish-blue and contains many, very fine, dustlike* reddish-like granules, as well as a few larger ones. The cytoplasm of myeloblasts and lymphoblasts is basophilic and contains no granules. *In patients with myeloblastic leukemia*, a few promyelocytes and more mature myeloid forms may be identified. In patients with lymphoblastic leukemia, most of the cells other than the blasts are readily recognizable lymphocytes. Only a few neutrophilic leukocytes and an occasional promyelocyte or myelocyte may be seen. Many monocytes may be present in the marrow of patients with monoblastic leukemia. Peroxidase stain may be of some value. The granules of promyelocytes and myelocytes and of some monocytes can be demonstrated with the peroxidase stain The azurophilic granules of lymphocytes do not give the reaction *Auer bodies*, reddish rod-shaped structures in the cytoplasm, are diagnostic of leukemia and are observed in monoblasts and myeloblasts, but not in lymphoblasts. Auer bodies are present, however, in only a small proportion of patients with myeloblastic or monoblastic leukemia.

Chronic Myelocytic Leukemia. Bone marrow examination is of little or no value in the diagnosis of this disease The preparations are usually very cellular and the M·E ratio is increased. There is *frequently, but not invariably*, a slight shift in the "maturation" curve to the more immature forms with myelocytes and metamyelocytes predominating (fig. 24). Basophilic and eosinophilic myeloid forms *are usually increased. Although bone marrow* examination may be of some value in ruling out myelofibrosis, the diagnosis of chronic myelocytic leukemia is made more easily from the blood *than from the marrow.*

Chronic Lymphocytic Leukemia. The diagnosis of this dis-

order, in most cases, is made more easily from the examination of the blood than from the bone marrow. The proportion of normal adult lymphocytes in the marrow is usually increased. The other constituents are little altered from the normal. Lymphoblasts are usually not increased significantly.

Infectious Mononucleosis. Examination of the bone marrow is rarely necessary or helpful in this disease. The only alterations are a modest increase in the proportion of myelocytes and the presence of a few abnormal lymphocytes, such as those which can be observed in the blood The diagnosis of infectious mononucleosis is based on (a) the clinical picture, (b) the serologic reaction, and (c) cytologic study of the blood. In rare and unusual patients, marrow examination may be necessary to rule out the possibility of acute leukemia.

Idiopathic Thrombocytopenic Purpura. The number of megakaryocytes is increased, particularly the more immature forms The adult megakaryocytes show degenerative changes in the nuclear chromatin and in the cytoplasm. Platelets attached to, and forming from, the cytoplasm of the adult megakaryocytes are reduced in number. In normal marrow, platelets can be seen forming from the cytoplasm of at least 50 per cent of the megakaryocytes. Platelet formation from the megakaryocytes of a patient with idiopathic thrombocytopenic purpura will be observed in less than 20 per cent of the cells. Unfortunately, however, none of these alterations distinguishes the secondary form of the disease from the primary variety, and examination of the bone marrow is of no value in the diagnosis of this disease, except as a means of ruling out acute leukemia. Although splenectomy is contraindicated in the absence of megakaryocytes in the marrow, the absence of megakaryocy- is more likely to be the result of a sampling error than a va observation.

REFERENCES

1 Sandoz Atlas of Hematology Basle, Switzerland, Sandoz Ltd (In the author' opinion this is the best atlas for the price It may be purchased from Sando Blood Atlas, 68 Charlton St, New York 14, N Y)
2. Leitner, S J, Britton, C J C, and Neumark, E Bone Marrow Biopsy New York, Grune & Stratton, 1949
3 Sundberg, R D Sternal aspiration Staff Meet. Bull Hosp Univ Minnesota 17 (26) 389, 1946

4. BERMAN, L.: A review of methods for aspiration and biopsy of bone marrow Am. J Clin Path 23: 385, 1953
5. SCHLEICHER, E. M: Isolation of particles from aspirated sternal marrow for biopsy Am J. Clin. Path. 17: 909, 1947.
6. ——: A new apparatus for isolation and preparation of aspirated bone marrow particles. Am. J. Clin Path. 20. 476, 1950.
7. BLOCK, M, SMALLER, V., AND BROWN, J An adaptation of the Maximow technique for preparation of sections of hemopoietic tissues. J. Lab. & Clin. Med 42. 145, 1953
8. SCHLEICHER, E. M.: An improved hematoxylin eosin stain for sections of marrow units Stain Technol. 28. 119, 1953
9. PEASE, G L.. Granulomatous lesions in bone marrow. Blood 11. 720, 1956.

Examination of Blood Pigments Related to Hemoglobin

As an introduction to the study of blood, urine, and stool pigments related to hemoglobin, the following brief and undoubtedly oversimplified summary of the biochemistry, physiology and pathology of bilirubin metabolism and jaundice is presented.

THE BIOCHEMISTRY OF JAUNDICE

Formation, Transportation and Excretion of Bilirubin. The senescent erythrocytes are taken from the circulation by the phagocytic reticuloendothelial system of the body. The spleen is particularly active in this respect. The degradation of hemoglobin to bilirubin takes place within the reticuloendothelial cells (fig. 27). The first step which occurs is the sequential oxidation of the α-methene bridge of the protoporphyrin ring. The ring is opened to form verdohemoglobin, which is a green biliverdin-globin compound. Iron is removed, globin liberated and the free biliverdin (green) is reduced at the γ-methene bridge to yield bilirubin (orange-red). The free iron combines with the iron-binding protein, transferrin, in the plasma and is transported to storage depots or to the bone marrow The globin is degraded and returned to the body pool of amino acids The free bilirubin passes out of the

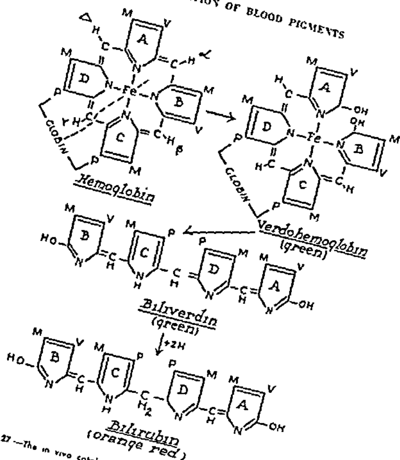


Fig 27.—The in vivo catabolism of hemoglobin to bilirubin

reticuloendothelial cells into the plasma In the plasma the bilirubin is bound loosely to albumin and is transported to the liver (fig. 28). Within the liver cells the bilirubin is conjugated with glucuronic acid. The type of linkage between glucuronic acid and bilirubin is not known with certainty. It has been suggested that the propionic acid side chains of bilirubin are linked with the C_1 hydroxyl of glucuronic acid. Be that as it may, under normal circumstances most of the bilirubin passes into the bile canaliculi as the soluble, bilirubin diglucuronide A small amount of conjugated bilirubin

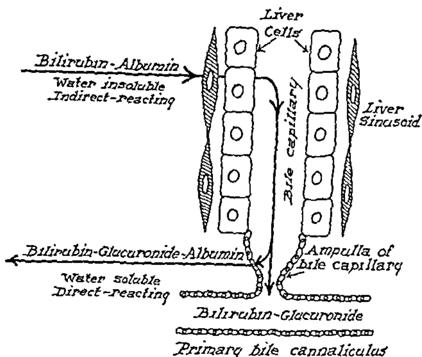


Fig 28—The van den Bergh reaction. Modified from Watson (Blood 1 99, 1946)

is normally regurgitated back into the plasma where it is again loosely attached to albumin Both mono- and diglucuronides of bilirubin are present in small quantities in the plasma.

The soluble bilirubin-glucuronides can be filtered readily by the kidneys. The relatively insoluble free bilirubin does not pass the glomerular barrier.

Bacterial Reduction of Bilirubin By The Intestinal Flora. In the intestinal tract, the bacteria remove the glucuronic acid from the bilirubin and the bilirubin is reduced in step-wise fashion to a number of bile pigments (fig. 29) which differ only in the degree of reduction. The degree of reduction of bilirubin and the compounds formed probably differ with the type of bacterial flora present. The term "urobilinogen" is used to refer collectively to the three colorless chromogens· d-urobilinogen, mesobilirubinogen, and stercobilinogen. Normally, sterocobilinogen is the preponderant

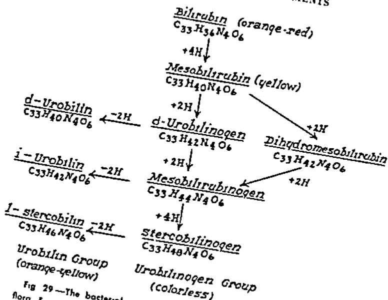


Fig 29—The bacterial reduction of bilirubin by the human intestinal flora From Watson (Tr A Am Physicians 67 242, 1954)

"urobilinogen" in feces and urine. The administration of wide-spectrum antibiotics to human subjects markedly diminishes the bacterial flora which reduce bilirubin, and under these conditions urobilinogen excretion decreases to very low levels and bilirubin is the predominant bile pigment. d-Urobilinogen has not yet been demonstrated in the feces of an individual who has not received a wide-spectrum antibotic. As the bacterial flora returns following the administration of an antibiotic, it is first capable of reducing bilirubin only as far as d-urobilinogen. Later, the full reducing capacity of the flora is restored and stercobilinogen again quantitatively becomes the predominate member of the urobilinogen group

Normally a small amount of the urobilinogen in the intestinal tract is reabsorbed and is re-excreted through the liver or appears in the urine. This is known as the enterohepatic circulation of urobilinogen and presumably accounts for the small amount of urobilinogen which is normally present in urine.

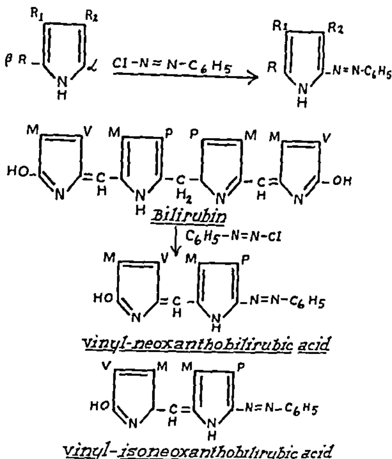


Fig 30 —The chemistry of the diazo reaction

When the urobilinogen group of compounds is exposed to air, they are partially oxidized to the urobilin group of compounds. Thus, when urine and feces are exposed to air, d-urobilinogen is oxidized to d-urobilin, mesobilirubinogen is oxidized to i-urobilin, and stercobilinogen to l-stercobilin.

The van den Bergh Test. In the van den Bergh reaction, bilirubin is coupled with diazotized sulfanilic acid (the diazo reagent) to form what is quite erroneously called "azobilirubin" (fig 30) The ability to couple with diazotized sulfanilic acid is possessed by pyrrolic compounds containing either a free α or β position. Pyrrolic

compounds without free α or β positions will couple only if the substituent group is readily displaced, such as a carboxyl group. Inspection of the bilirubin formula reveals that neither α or β positions are free and the hydroxyl groups cannot be readily replaced. Therefore, it has been suggested that in the reaction of diazotized sulfanilic acid with bilirubin, the molecule is split into two dipyrrol compounds, vinyl-neoxanthobilirubic acid and vinyl-isoneoxanthobilirubic acid (fig. 30). These two compounds have free α positions and they diazotize readily.

Bilirubin and mesobilirubin are the only naturally occurring bile pigments which react with diazo reagent.

Van den Bergh and Miller recognized that the formation of azobilirubin differed in the various forms of jaundice. "Azobilirubin" formed simply on the addition of the diazo reagent to the serum of patients with obstructive jaundice On the other hand, the addition of substances containing alcoholic groups (methyl or ethyl alcohol) was essential for the reaction to take place with normal sera and with sera from patients with hemolytic jaundice. The reaction which takes place without the addition of alcohol has been referred to as the direct van den Bergh reaction. That which takes place only in the presence of alcohol has been called the indirect van den Bergh reaction It is now known that the soluble mono- and diglucuronides of bilirubin account for the direct reaction. The relatively insoluble free bilirubin reacts with the diazo reagent only after it is taken into solution with alcohol (fig 28).

The Ehrlich Aldehyde Reaction. When the Ehrlich aldehyde reagent (p-dimethylaminobenzaldehyde in acid solution) is added to certain bile pigments a red color develops. The chemical nature of this reaction is not understood. Among the naturally occurring bile pigments, d-urobilinogen, mesobilirubinogen, and stercobilinogen are the only ones which will react with the Ehrlich aldehyde reagent Bilirubin, mesobilirubin and the urobilin group of compounds will not react. Porphobilinogen, the colorless monopyrrolic compound which is excreted in the urine under certain conditions, will react with the aldehyde reagent (p. 228).

Intravascular Hemolysis. When red cells are destroyed within the vascular channels, the catabolism of hemoglobin proceeds in

quite a different fashion than it does when the red cells are destroyed within the reticuloendothelial system (fig. 31). During intravascular hemolysis free hemoglobin gains access to the plasma where it is immediately bound to the hemoglobin-binding protein, haptoglobin (Hp). One molecule of haptoglobin, an α_2-mucoprotein, is capable of binding two molecules of hemoglobin. The entire $HpHb_2$-complex is removed from the plasma at a constant rate by the reticuloendothelial system. The hemoglobin is then degraded into bilirubin in the usual fashion within the reticuloendothelial cells.

When the concentration of free hemoglobin in the plasma exceeds the binding capacity of haptoglobin, free hemoglobin is found in the plasma. The free hemoglobin is rapidly oxidized to methemoglobin and methemalbumin. These three compounds, free hemoglobin, methemoglobin and methemalbumin are then eliminated into

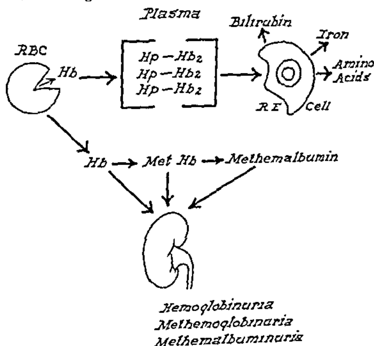


Fig 31.—The metabolic pathways of free hemoglobin resulting from the intravascular hemolysis of red cells
Hb = hemoglobin, $HpHb_2$ = haptoglobin-hemoglobin complex; RE cell = reticuloendothelial cell

the urine. Since the molecular weight of the haptoglobin hemoglobin complex ($HpHb_2$) is about 310,000, the molecule does not pass into the urine. Only when more hemoglobin has been liberated into the plasma than can be bound by haptoglobin, does hemoglobinuria appear.

THE PATHOPHYSIOLOGY OF JAUNDICE

Jaundice is the term applied to the yellow pigmentation occurring in the various tissues and is associated with an increase in the bilirubin content of the plasma. Normally, the plasma contains less than 1.5 mg. of bilirubin per 100 ml. When the plasma bilirubin reaches concentrations of about 2.0 mg./100 ml. the yellow pigmen-

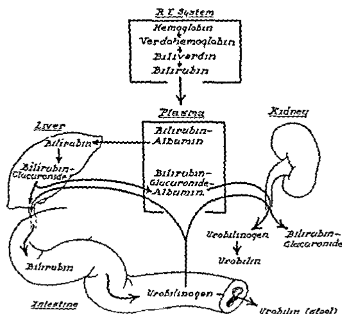


Fig. 32.—The metabolic pathways of bilirubin

tation of the body tissues (particularly the sclerae) can, under favorable circumstances, be detected by the skilled observer; with

concentrations above 5 to 7 mg./100 ml. jaundice is obvious to even the unskilled.

From a simple consideration of figure 32, it is apparent that jaundice may result from one of three general situations, or some combination thereof: (1) when bilirubin is presented to a normal liver at a rate which exceeds the ability of the liver to conjugate and excrete the pigment into the biliary system; (2) when the liver cells are functionally impaired and they cannot conjugate and excrete a normal amount of bilirubin presented to them; and finally (3) when the free flow of conjugated bilirubin through the biliary system is impaired. From these considerations jaundice can be conveniently classified as (1) prehepatic (hemolytic jaundice), (2) hepatic jaundice (parenchymal, hepatocellular), and (3) posthepatic (obstructive) (table 54).

TABLE 54.—*Classification of Jaundice*

I. Prehepatic (Hemolytic disease)

II. Hepatic
- A. *Hepatocellular*
  1. Constitutional hyperbilirubinemia
  2. Hepatitis (infectious and serum)
  3. Cirrhosis (alcoholic, hemochromatosis, Wilson's disease)
  4. Toxins (bacterial)
  5. Poisons (arsphenamine, cinchophen, carbon tetrachloride, phosphorus, trinitrotoluene, sulfanilamide)
  6. Chronic passive congestion
- B. *Hepatocanalicular*
  1. Primary biliary cirrhosis
  2. Secondary biliary cirrhosis (hepatitis, drugs)

III. Posthepatic
- A. Benign (usually incomplete obstruction)
  1. Stones
  2. Strictures
- B. Malignant (frequently complete obstruction)
  1. Carcinoma of pancreas
  2. Carcinoma of ampulla of Vater
  3. Carcinoma of gallbladder
  4. Carcinoma of bile duct or hepatic duct

In prehepatic jaundice (hemolytic disease) the fundamental abnormality is an increased rate of destruction of red cells, and, consequently, an overproduction of bilirubin. The amount of bili-

rubin produced per unit of time exceeds the rate at which the liver can clear it from the blood with the result that it accumulates in the plasma. The plasma bilirubin is mainly of the indirect type in the van den Bergh test and the excretion of urobilinogen in both feces and urine is increased. Since the bilirubin present in the plasma is in the insoluble form as free bilirubin, it is not filtered by the kidneys and bilirubinuria is absent.

In hepatic jaundice the fundamental defect is an inability of the liver cells to conjugate or to excrete bilirubin at the normal rate. In patients with constitutional hyperbilirubinemia, the liver is deficient in the enzyme system which is involved in the conjugation of bilirubin. As a consequence, the bilirubin in the plasma is almost entirely in the free form (indirect-reacting) and bilirubinuria is absent. The amount of urobilinogen found in the urine and feces is within normal limits since the overall rate of hemoglobin catabolism is within normal limits. In patients with hepatocellular disease both the free and the conjugated bilirubins are increased in the plasma (p. 221). The fecal urobilinogen is usually within the normal range, the amount of urobilinogen in the urine is frequently increased and bilirubinuria may be present. In the hepatocanalicular type of jaundice the liver cells are unable to excrete bilirubin at the normal rate and passage of bilirubin in the bile canaliculi is retarded The result is that there is a considerable "regurgitation" of conjugated bilirubin into the plasma and bilirubin is excreted in the urine. The amount of urobilinogen in the feces may be normal or decreased The amount of urobilinogen in urine is usually within normal limits.

In posthepatic jaundice the fundamental defect is in the passage of conjugated bilirubin through the biliary system. If this passage is completely blocked, conjugated bilirubin will be "regurgitated" into the plasma and excreted into the urine There will be an absence of urobilinogen in the stools. Since there is little or no urobilinogen in the gastrointestinal tract to be reabsorbed into the enterohepatic circulation, little or none will be found in the urine. There is one special circumstance worthy of mention. Patients with complete biliary obstruction and cholangitis or cholangiolitis may be found to have urobilinogen in the urine but not in the feces The explanation for this is that bacteria proximal to the obstruction reduce bilirubin to urobilinogen and the urobilinogen is "re-

gurgitated" into the plasma and excreted in the urine.

In subjects with no abnormality in the conversion of bilirubin to urobilinogen or in the ability to excrete urobilinogen, the total amount of urobilinogen excreted per day is directly related to the number of grams of hemoglobin catabolized per day. One gram of hemoglobin is equivalent to 37 mg. of urobilinogen on a stoichiometric basis. Assuming that the subject is in hematologic equilibrium, that is, the amount of hemoglobin produced per day is the same as the amount destroyed per day, then the amount of urobilinogen produced and excreted per day is a function of (1) the size of the total circulating hemoglobin pool (TCH)and (2) the red cell survival time (ST). This relationship is shown in the following formula:

$$\frac{\text{TCH (Gm.)}}{\text{S.T. (days)}} \times 37 \text{ mg.} = \text{Urobilinogen production in mg./day}$$

The total amount of circulating hemoglobin (TCH) is related to (1) the total blood volume (B.V.) and (2) the concentration of hemoglobin (Hb). This relationship is shown in the following formula:

$$\frac{\text{B.V. (ml.) x Hb (Gm./100 ml.)}}{100} = \text{TCH in grams}$$

The blood volume is related to the size of the individual. For rough calculations, it may be assumed that the blood volume is approximately 65 ml /Kg. of body weight.

From the above quantitative considerations it is possible to derive a hemolytic index. This index, if not over interpreted, may be helpful in the study of a patient with jaundice

The expected urobilinogen excretion is calculated by assuming that the erythrocyte survival time is normal (120 days). This is done as follows:

(1) Body weight in Kg. x 65 ml. = Blood volume in ml.

(2) $\frac{\text{B.V. (ml.) x Hb (Gm 100 ml.)}}{100}$ = T.C.H. in Gm

(3) $\frac{\text{T.C.H. x 37}}{120}$ = Expected daily urobilinogen excretion in mg.

To calculate the hemolytic index (H.I.) the actual amount of urobilinogen which has been found to be excreted is divided by the expected urobilinogen excretion, assuming the red cell survival time to be 120 days Thus,

$$\frac{\text{Determined Urobilinogen}}{\text{Expected Urobilinogen}} = \text{H.I.}$$

An index of one or less suggests that the red cell survival time is normal, and, therefore, hemolytic disease is not the explanation for the jaundice. If the index is considerably more than one (two or more), it is likely that a hemolytic disease is present.

In table 55 several examples of these calculations are given to illustrate how such calculations are useful. For example, patients 1, 2 and 3 each excreted the same amount of urobilinogen, 250 mg./day This is within the normal range of 40 to 280 mg However, in case number 2, when consideration is given to the small size of the total circulating pool, the excretion of urobilinogen is more than three times that which would be expected. In case 3, when consideration is given to the small size of the individual as well as the great reduction in total circulating hemoglobin, the excretion of 250 mg. of urobilinogen is 8 times the expected amount. On the other hand, although case 4 excreted more urobilinogen than did case 3, this was no more than expected when consideration is given to the large body size and the large total circulating hemoglobin pool. Case number 5 is an example of a severe hemolytic anemia

TABLE 55.—*Calculation of the Hemolytic Index*

Case No	Body Weight Kg	Hb Conc Gm /100 ml	Urobilinogen, Determined mg /day	Urobilinogen Expected* mg /day	Hemolytic Index
1	70	16	250	224	1.1
2	70	5	250	70	3.6
3	30	5	250	30	8.3
4	100	17	350	341	1.0
5	70	10	1554	140	11.1

*Assuming a normal erythrocyte survival time of 120 days

Presumptive Tests

Place 15 ml. of blood in a centrifuge tube containing an anticoagulant such as 20 per cent potassium oxalate. Centrifuge the freshly drawn blood and inspect the plasma to determine if the abnormal pigment is intracellular or extracellular.

The important abnormal intracellular pigments are carbon monoxide hemoglobin (carboxyhemoglobin), methemoglobin, and sulfhemoglobin. The important extracellular pigments are bilirubin, hemoglobin, and methemalbumin. In rare circumstances methemoglobin may appear in the plasma. Sulfhemoglobin is entirely an intracellular pigment and does not appear in the plasma.

If the plasma is yellow, bilirubin is present. If there is hemoglobinemia, the plasma will be pink or red. If the plasma is brown, it is likely that methemalbumin or methemoglobin or both are present. If an abnormal pigment is observed in the plasma, decant or pipet off the plasma and wash the erythrocytes twice with physiologic saline. If an abnormal pigment is present in the plasma, it is possible that an abnormal intracorpuscular pigment is also present, and both the plasma and the cells must be examined separately.

If the plasma is clear, shake the whole blood in the air for 15 minutes. Fully oxygenated blood is bright red; blood containing carbon monoxide hemoglobin is cherry-red; blood containing methemoglobin or sulfhemoglobin is a chocolate-brown color. However, unless the concentration of the abnormal hemoglobin derivative is high, the color will be masked by the oxyhemoglobin and more specific tests must be performed.

Carbon Monoxide Hemoglobin

Dilution Test

In dilute solutions oxyhemoglobin appears yellowish-red while carbon monoxide hemoglobin appears pinkish or bluish-red. Add a drop of normal blood to 5 ml. of water and make a series of dilutions. Make a parallel series of dilutions of the unknown until by comparison a difference in tint is noted.

Alkali Test

Place two drops of normal blood and two drops of the unknown blood side by side on a spot plate. Add two drops of 25 per cent sodium hydroxide to each. Normal blood turns a brownish color. If carbon monoxide hemoglobin is present the original reddish color will persist.

Methemoglobin and Sulfhemoglobin

Dilute the whole blood or washed erythrocytes with water 1:10 or 1:100 depending upon the amount of abnormal pigment present and place a few ml. in each of four test tubes. Since methemoglobin is formed spontaneously in normal blood, especially when diluted, it is imperative to examine the blood without delay after the dilution is made.

Tube 1. Examine in the hand spectroscope. If methemoglobin is present, a dark band will be visible at 630 mμ. If sulfhemoglobin is present, a dark band will be visible at 618 mμ. These bands cannot, however, be differentiated in the hand spectroscope. Keep this tube for a reference.

Tube 2. Add two or three drops of a 5 per cent solution of potassium cyanide (Do not use a pipet. Cyanide is extremely toxic if ingested.) If the band is due to methemoglobin, it will disappear immediately, differentiating it from the sulfhemoglobin band which remains fixed.

Tube 3. Add two or three drops of hydrogen peroxide (3 per cent). If the band is due to either methemoglobin or sulfhemoglobin, it will disappear.

Tube 4. Add 0.1 ml of concentrated ammonium sulfide per ml. of diluted blood. If the band is due to methemoglobin, it will disappear. The blood must be examined shortly following the addition of the sulfide since on standing, sulfhemoglobin forms and a band will reappear at 618 mμ. If the band is due to sulfhemoglobin, it will remain unaltered.

Methemalbumin

Centrifuge the whole blood and pipet 2 ml. of plasma into each of 4 small test tubes (Wasserman tubes).

Tube 1. Examine in the hand spectroscope. If a band is observed at 576 mμ, hemoglobin is present. If a band is observed in the region of 620 to 630 mμ the following test must be performed to differentiate methemalbumin from methemoglobin. Keep this tube for a reference.

Tube 2. Add two drops of a 5 per cent solution of potassium cyanide (Do not pipet the cyanide solution. Use a dropper). If methemalbumin is present the band will remain fixed. If methemoglobin is present, the band will disappear, and the specimen will change color from brown or black to dark red.

Tube 3. Add two drops of hydrogen peroxide (3 per cent) and shake. If methemalbumin is present, the band at 624 mμ will remain fixed. If methemoglobin is present the band will disappear.

Tube 4. Add 0.2 ml. of concentrated ammonium sulfide. If methemalbumin is present, the band in the region of 624 mμ will disappear and a dense easily visible band will appear at 558 mμ. Occasionally, an absorption band will not be visible in the region of 624 mμ but, on the addition of ammonium sulfide, a band will appear at 558 mμ. This procedure is known as Schumm's test and it is a valuable test when performed along with the cyanide and hydrogen peroxide tests. It is not, however, specific for methemalbumin since methemoglobin gives a similar reaction. The plasma must be examined immediately after the addition of the sulfide since, on standing, sulfhemoglobin forms, and a band reappears at 618 mμ.

REFERENCES

1 FINCH, C A Methemoglobinemia and sulfhemoglobinemia New England J. Med *239* 470, 1948

2 ROSS, J F Hemoglobinemia and the hemoglobinurias New England J Med. *233* 691, 732, 766, 1945

3 FAIRLEY, N H · Methemalbumin Quart. J Med *10* 95, 115, 1941

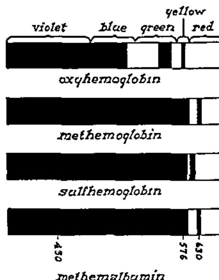


Fig 33 —The absorption spectra of several important blood pigments

TABLE 56—*Differential Reactions of the Blood Pigments*

Pigment	Alpha Band mμ	Potassium Cyanide 5%	Hydrogen Peroxide 3%	Ammonium Sulfide Conc
Oxyhemoglobin	576			
Methemoglobin	630	Disappears	Disappears	Disappears
Sulfhemoglobin	618	Unaltered	Disappears	Unaltered
Methemalbumin	624	Unaltered	Unaltered	Disappears

Total Heme Pigments

Collection of Blood

1. Draw blood into a syringe which has been lightly coated with mineral oil The syringe must be scrupulously clean. The veni-

puncture should be performed by entering the vein without difficulty and free-flowing blood obtained. The syringe should be allowed to fill by venous pressure and without the use of suction.

2. Remove the needle from the syringe and allow 5 ml. of blood to run gently down the side of a centrifuge tube containing 200 units of heparin (0.1 ml. of Liquaemin-Roche-Organon).

3. Mix the contents of the tube by gently rolling the tube between the palms of the hands.

4. Centrifuge the tube for 10 minutes at 1500 R.P.M.

5. Dilute 1 ml. of the plasma with distilled water until the color is a faint pink.

Technic

1. Set up 3 test tubes (15 ml.) and add 1 ml. of the benzidine solution to each tube.

2. Add 0.02 ml. (Sahli hemoglobin pipet) of the unknown plasma to tube 1 (unknown), 0.02 ml. of distilled water to tube 2 (blank), and 0.02 ml. of the dilute standard hemoglobin solution to tube 3 (standard). Mix the contents of the tube.

3. Add 1 ml. of the hydrogen peroxide solution to each tube and mix immediately.

4. When the color change (green to blue to violet) has been completed (approximately 20 minutes), add 10 ml. of the diluent solution to each tube and mix by inverting the tubes.

5. After 10 minutes transfer the 3 solutions to cuvettes. Set the photometer at a wave length of 515 mμ or insert the appropriate filter. Set the blank tube to read 0 on the density scale or 100 per cent on the transmission scale. Read the standard and then the unknown.

Calculation

1. If the galvanometer scale is in per cent transmission, convert the readings to density by the use of a table relating density to per cent transmission.

2. The plasma hemoglobin concentration in mg./100 ml. is calculated from the formula $\frac{D_u}{D_s} \times A \times$ dilution of unknown, where

D_u is the density of the unknown, D_s is the density of the standard, and A is the concentration of hemoglobin (mg./100 ml.) in the dilute standard. For example, if the concentration of the concentrated hemoglobin standard is 10 Gm./100 ml.; the density reading of the standard tube, 0.310; the density reading of the unknown, 0.350, and the plasma was diluted 1:10; then the concentration of the dilute standard is $\frac{0.02}{10} \times 10 = 0.02$ Gm./100 ml., or 20 mg./100 ml Then, $\frac{0.350}{0.310} \times 20 \times 10 = 22$ mg./100 ml.

Reagents

Benzidine solution (1 per cent)
Benzidine base 1 Gm.
Glacial acetic acid 90 ml.
Distilled water to make 100 ml

This reagent should be kept in the refrigerator in a dark bottle and made up fresh every two weeks

Hydrogen peroxide solution (1 per cent)
Hydrogen peroxide (3 per cent) 10 ml
Distilled water 20 ml.

This solution should be stored in a dark bottle in the refrigerator and made up fresh every two days. Care should be taken to avoid peroxide which has been stabilized with sulfuric acid because sulfate precipitates benzidine.

Diluent solution
Glacial acetic acid 10 ml.
Distilled water to make 100 ml.

Concentrated hemoglobin standard

Wash 2 ml. of packed red cells with physiologic saline and lyse them by freezing and thawing Dilute the hemoglobin with physiologic saline to a concentration of about 10 Gm. of hemoglobin /100 ml Determine the hemoglobin concentration by the photometric method (p. 27).

Dilute hemoglobin standard

Dilute 0.02 ml. (Sahli hemoglobin pipet) of the concentrated standard to 10 ml. with physiologic saline.

Interpretation

1. If the blood is properly collected the "normal" level of heme pigment in plasma is less than 5 mg./100 ml.

2. Levels of 50 to 1000 mg./100 ml. may occur in patients with intravascular hemolysis.

Comments

1. All glassware must be cleaned carefully in cleaning solution, rinsed thoroughly with distilled water and air dried.

2. The distilled water must be free of sulfate. This can be checked by the addition of several drops of a solution of barium chloride. If no cloudiness appears, sulfates are absent.

3. Great care must be taken to avoid hemolysis of the blood due to trauma.

4. This method measures total heme pigments (methemoglobin, methemalbumin) in the plasma and not just hemoglobin.

5. When the concentration of hemoglobin in the plasma is high, it may be measured directly as cyanmethemoglobin (p. 27).

6. When the concentration of the plasma hemoglobin is low, the accuracy of the method may be increased in several ways. The strength of the benzidine solution may be increased to 4 per cent. The volume of the unknown may be increased to 0.05 ml. The volume of the diluent may be reduced to 4 ml. in place of 10 ml. It is desirable to adjust the concentration so that the reading falls between 0.1 and 0.6 density or between 10 and 90 per cent transmission

7. This method may be used for the quantitative determination of hemoglobin in urine. The salts in urine cause turbidity and interfere with color development in the benzidine reaction This may be overcome by dialyzing the urine overnight against physiologic saline. The dilute standard hemoglobin solution should also be dialyzed. The volume of both solutions is measured before and after dialysis and appropriate corrections are made for the change in volume. As much as 1 ml. of dialyzed urine may be used in the determination by decreasing the volume of diluent accordingly.

8. The reagent blank should be almost colorless If it is highly colored, reagents or glassware have been contaminated with heme pigments.

9. The accuracy and reproducibility of the method is about ± 5 per cent.

REFERENCES

1. Crosby, W. H and Dameshek, W.: The significance of hemoglobinemia and associated hemosiderinuria, with particular reference to various types of hemolytic anemia. J. Lab & Clin Med 38, 829, 1951.
2. Ham, T. H.: Hemoglobinuria Am J. Med. 18 990, 1955
3. Crosby, W. H. and Furth, F. W.: A modification of the benzidine method for the measurement of hemoglobin in plasma and urine Blood 11 380, 1956

Bilirubin

Technic

1. Dilute 1.0 ml. of serum or plasma to 20 ml with distilled water.
2. Transfer 5 ml. of the diluted serum to each of 2 clean cuvettes.
3. Add 1.0 ml. of the diazo blank solution to tube 1.
4. With filter 510 and the 6 ml. aperture of the Evelyn photometer, adjust the instrument so that the blank tube (tube 1) reads 100 per cent transmission on the galvanometer scale.
5. Add 1.0 ml. of the diazo reagent to tube 2.
6. Exactly one minute after the addition of the diazo reagent, read tube 2. This gives the prompt direct reacting (1 minute) reading.
7. Add 6 0 ml. of absolute methyl alcohol to each of the 2 tubes
8. Fifteen minutes later, set the galvanometer scale at 100 per cent transmission with tube 1 (blank) in place and then read tube 2 (unknown). This gives the total bilirubin reading.

Comments

1. The above procedure is designed for the Evelyn photometer but may be readily adapted to other photometers. A number 54 filter is used with the Klett-Summerson instrument. If a spectrophotometer is used, the determination is made at a wave length of 540 mμ and if the scale reads directly in density, the blank tube is set at O density with the slit adjustment control

2. If the serum is quite icteric, it should be diluted 1:100 rather than 1:20. It is always desirable, if possible, to make a dilution such that the reading on the galvanometer scale is between 10 and 90 per cent transmission.

Preparation of a Standard Curve

1. By the use of an analytical balance weigh out accurately about 10 mg. of pure bilirubin.*
2. Quantitatively transfer the bilirubin to a 100 ml. volumetric flask. Make up to volume with chloroform (C.P.).
3. With a 10 ml. volumetric pipet, pipet exactly 10 ml. of the concentrated standard into a second 100 ml. volumetric flask. Make up to volume with absolute methyl alcohol.
4. Pipet 2, 4, 6 and 8 ml. of the dilute standard into each of 4 cuvettes. Add 1.0 ml. of freshly prepared diazo reagent to each cuvette and to a "blank" cuvette. Add sufficient absolute methyl alcohol to each tube to make a final volume in each tube of 10 ml
5. With the blank tube in place adjust the galvanometer to read 100 per cent transmission.
6. Fifteen minutes after the addition of the diazo reagent to the standard solutions read the tubes in the instrument.
7. The concentration of azobilirubin in a solution is proportional to the negative logarithm of the light transmission Therefore, the following formula applies, $C = \frac{2 - \log G}{K_1}$ where C is the concentration of azobilirubin (expressed as bilirubin) in mg. per ml. of colored solution, G is the galvanometer reading and K_1 is the constant.

The value for K_1 is calculated for each of the four standard solutions. The mean of the four values is taken.

Interpretation

1. Conversion of the bilirubin concentration in terms of mg. per ml. of colored solution to mg. per 100 ml. of serum is made by

*Eastman Kodak, Rochester, N Y or Hoffman La Roche, Nutley, N J

means of the formula $X = \frac{2 - \log G}{K_1} \times \frac{V}{A} \times 100$ where X is mg. of bilirubin per 100 ml. of serum, V is the total volume of the solution in the cuvette, and A is the amount of serum used

2. Bilirubin is normally conjugated with glucuronic acid in the liver and is excreted in this form in the bile. Bilirubin glucuronide is water soluble and, therefore, is able to react promptly and directly with the diazo reagent Unconjugated bilirubin is water insoluble and reacts with the diazo reagent only after it is solubilized by alcohol

3. The mean value (± 2 S D.) for the direct reacting bilirubin in normal human subjects is 0.11 ± 0.10 mg /100 ml. Values above 0.25 mg./100 ml. may be considered abnormal. The mean normal value (± 2 S.D.) for the total serum bilirubin is 0.62 ± 0.50 mg./100 ml. Values above 1.50 mg /100 ml may be considered abnormal

4 In cases of early hepatitis, cirrhosis, common duct stone and hepatic carcinomatosis, the direct reacting (1 minute) bilirubin fraction may be increased significantly within a normal value for total bilirubin.

5. The ratio of the direct reacting bilirubin (1 minute) to the total serum bilirubin (T), $\frac{D}{T} \times 100$, is useful clinically. Ratios below 20 per cent are characteristic of prehepatic jaundice (hemolytic disease) and constitutional hyperbilirubinemia If the ratio is less than 40 per cent and is associated with a significant increase in total bilirubin the likelihood is that the cause of the jaundice is prehepatic or hepatic (parencymal) and not posthepatic (biliary obstruction). In posthepatic jaundice the ratio is between 45 and 80 per cent However, the ratio will be this high in many cases of hepatic jaundice. Thus, ratios above 50 per cent are of no significance in distinguishing hepatic jaundice from posthepatic jaundice

Reagents

Solution A

Sulfanilic acid 1.0 Gm.

Concentrated hydrochloric acid 15 ml.
Make up to 1 liter with distilled water

Solution B

Sodium nitrite 0.5 Gm.
Distilled water to make 100 ml.

Diazo Reagent

Solution A, 10 ml.
Solution B, 0.3 ml.

Diazo Blank

Concentrated hydrochloric acid 15 ml.
Distilled water to make 1 liter

Solution B and the diazo reagent must be made before use.

REFERENCES

1. MALLOY, H T AND EVELYN, K. A: The determination of bilirubin with the photoelectric colorimeter. J. Biol Chem. *119*. 481, 1937.
2. ZIEVE, L, HILL, E, HANSON, M, FALCONE, A B., AND WATSON, C. J: Normal and abnormal variations and clinical significance of the one-minute and total serum bilirubin determinations J. Lab & Clin. Med. *38*. 446, 1951.
3. WATSON, C J.. The importance of the fractional serum bilirubin determination in clinical medicine. Ann Int. Med *45*: 351, 1956

Examination of Urine Pigments Related to Hemoglobin

Urobilinogen

Technic

1. A two-hour urine specimen is collected, preferably from 2 to 4 p.m. The patient voids at the beginning of the period, discards the urine, drinks a glass of water, and voids two hours later. The volume of the latter specimen is recorded, and the Ehrlich reaction is measured within one-half hour after voiding.

2. Pipet 1 25 ml. of urine into each of the two 5 ml tubes accompanying the comparator block.

3. To one tube add 1.25 ml. of Ehrlich's reagent, mix the contents by inversion, and approximately 15 seconds later, add 2.5 ml. of a saturated aqueous solution of sodium acetate and mix thoroughly.

4. To the other tube, which is to serve as the blank, the reagents are added to the urine in reverse order to prevent color development. First 2.5 ml. of the solution of sodium acetate is added and the contents are mixed thoroughly. With constant shaking, slowly add 1.25 ml. of Ehrlich's reagent.

5. The blank and reaction tubes are placed in two adjacent openings in the comparator block and the sliding scale of dye standards is adjusted as necessary to bring the standard first selected for matching behind the blank and a vial of water behind the unknown tube. If the match of colors obtained by holding up the set in front of a light is not satisfactory, a stronger or weaker color standard is moved into position behind the blank until the best match is found. Interpolation may be made if it is evident that the color intensity lies between the two standards. If the blank develops a pink color or if the intensity of the unknown is higher than that of the color standard corresponding to a urobilinogen concentration of 0.6 mg. per 100 ml., further dilutions of the urine should be made, that is, 1:2, 1:4, or 1:8 and so on.

6. The total amount of urobilinogen for the two-hour period is calculated as follows:

$$\underset{(\text{mg./100 ml.})}{\text{Standard Reading}} \times 4 \times \frac{\text{ml. urine excreted in 2 hrs}}{100} \times \text{any further dilution}$$

$$= \text{Ehrlich Units per 2 hours}$$

Example. Standard used in matching, 0.30 mg./100 ml.; two-hour urine volume 85 ml.; no additional dilution.

$$0.30 \times 4 \times \frac{85}{100} = 1.0 \text{ Ehrlich units/2 hours.}$$

Precautions

1. The urine must be cooled to room temperature and analyzed within 30 minutes after voiding. If the urine is allowed to stand

for a longer period of time, the urobilinogen is oxidized to urobilin, a compound which does not react with Ehrlich's aldehyde reagent.

2. The saturated solution of sodium acetate must be added to the unknown tube approximately 15 seconds after the urine has been shaken with Ehrlich's reagent. The reason for this is that the color of the Ehrlich aldehyde reaction, as developed in the urine, deepens gradually as time elapses from the onset of the reaction, *and this deepening in color with time is due to nonurobilinogen reacting substances.*

3. The sodium acetate used must be pure, and the solution must be saturated (*crystals of sodium acetate should be present in the* bottom of the bottle). This is of particular importance insofar as the blank solution is concerned since if the hydrochloric acid is not fully changed to acetic acid a partial Ehrlich reaction will ensue, and the resultant value in Ehrlich units will be too low. If the sodium acetate is not pure, the color developed may fade rapidly with time.

4. *After the color has been developed, the solution should be* read within five minutes. Fading may occur after this time even when pure sodium acetate is used.

5. Bilirubin, if present, will interfere (high values) with the *determination of urobilinogen. If the degree of bilirubinuria is not* great the determination can be carried out satisfactorily by first *diluting the urine with 2* volumes of distilled water. *If* there are large amounts of bilirubin the urine should be extracted with an equal volume of a 10 per cent aqueous solution of barium chloride and filtered through a hard filter to remove all precipitate. Porphobilinogen, which is present in the urine of patients with acute intermittent porphyria, produces a pink aldehyde compound with Ehrlich's reagent. These two substances can be differentiated by adding a few ml. of chloroform *to the reaction tube. The aldehyde* compound of porphobilinogen remains in the aqueous fraction *while that of urobilinogen is extracted by* the chloroform

6. The method is applicable to the 24-hour urine provided the following rules for the collection of urine are adhered to strictly. The urine must be collected in a dark bottle to which 5 grams of sodium carbonate and about 25 ml of petroleum ether are first added. If these precautions are not *followed, the urobilinogen will* be oxidized to urobilin which does not react with the aldehyde reagent

7. The Ehrlich reagent should not be allowed to stand in the reaction tube in the comparator block after the reading has been made. Ehrlich's reagent contains hydrochloric acid and the fumes of acid will dissolve the metal parts and cause cloudiness of the glass in the window. The window glass and the glass vials must be kept clean at all times.

Interpretation

This method is not specific for urobilinogen and includes other Ehrlich reacting substances. The increases in the latter, however, are roughly proportional to that of the urobilinogen, so that from the clinical standpoint the information gained is usually comparable to that obtained by the exact quantitative method. The simplified method outlined above may be expected to give false negative results in about 15 per cent of patients with a significant degree of urobilinogen in the urine In normal subjects the total amount for the two-hour sample varies from 0.1 to 1.5 Ehrlich units Values between 1.5 and 2.5 units should be further evaluated by obtaining serial determinations on successive days. If interpretation is still in doubt, the *per diem* excretion of urobilinogen should be determined for at least one 24-hour period by means of the more elaborate 24-hour method.

It must be remembered that aureomycin and other wide-spectrum antibiotics cause the disappearance of the organisms from the bowel which normally convert bilirubin to urobilinogen Therefore, no reliance can be placed on the urobilinogen studies when these agents are being given. The change occurs within the first 48 to 72 hours of drug therapy and normal concentrations of urobilinogen may not be restored for as long as six or seven days following the discontinuance of drug administration.

Representative values in various types of jaundice are given in table 57

Reagents

Ehrlich's aldehyde reagent

Para-dimethylaminobenzaldehyde 0.7 Gm
Concentrated hydrochloric acid 150 ml.
Distilled water to make 100 ml
Store in a brown glass bottle with a glass stopper.

TABLE 57.—*Bile Pigment Values in Various Types of Jaundice*

Determination	Normal	Prehepatic	Hepatic			Posthepatic	
		Hemolytic	Constitutional Hyperbilirubinemia	Hepato Cellular	Hepato-Canalicular	Incomplete	Complete
Urine bilirubin	0	0	0	++	+++	+++	++++
Urine urobilinogen E U /2 hrs.	0 1–1.5	5–20	0 1–1.5	0 1–10	0–1	0 1–5	0–0.2
Stool urobilinogen E U /100 Gm	50–300	400–2000	50–300	10–200	0–200	10–200	0–5

The ranges given are representative and are not absolute Repeated determinations are more valuable than a single determination

Saturated aqueous solution of sodium acetate

Sodium acetate ($3H_2O$) C.P. 100 Gm

Distilled water to make 100 ml.

Heat to 60 C. and allow to cool to room temperature

A large excess of crystals should be present in the bottom at all times.

Urobilinogen standards and comparator blocks

The comparator block is manufactured by W. A. Taylor Co., Baltimore, Md., and can be purchased from the Will Corporation, Rochester, N. Y. The Will Corporation supplies the comparator blocks filled with vials of dye standards and water. The accessory pipets, rubber bulbs, cork stoppers and wide-mouth bottles are procurable from this source. The solutions of dye are stable, and if contamination and unnecessary exposure to light are avoided, the color densities should remain unchanged indefinitely.

REFERENCES

1 SCHWARTZ, S., SBOROV, V., AND WATSON, C J Studies of urobilinogen IV The quantitative determination of urobilinogen by means of the Evelyn photoelectric colorimeter Am J Clin Path 14 598 1944
2 WATSON, C J, SCHWARTZ, S, SBOROV, V, AND BERTIE, E Studies of urobilinogen V A simple method for the quantitative recording of the Ehrlich re

action as carried out with urine and feces Am. J. Clin Path 14 605, 1944.

3 ——— and Hawkinson, V. Studies of urobilinogen VI Further experience with the simple quantitative Ehrlich reaction Corrected calibration of the Evelyn colorimeter with a pontacyl dye mixture in terms of urobilinogen Am J Clin Path 17: 108, 1947

4 Young, L. E., Davis, R. W., and Hogestyn, J. Simplified equipment for determination of urobilinogen in urine and stool J. Lab. & Clin Med. 34, 287, 1949

Bilirubin

Technic

1. Place 5 drops of urine on one square of the test mat
2. Place one reagent tablet on the area of the mat moisten by the urine.
3. Flow two drops of water on the tablet allowing them to ru down over the side of the tablet.
4. Read 30 seconds later.

Comments

1. The reagent tablet contains p-nitrobenzenediazonium-p-toluene-sulfonate 0.2 mg, sulfosalicylic acid 100 mg, sodium bicarbonate 10 mg, and boric acid 20 mg The stable diazonium compound couples with bilirubin to give a blue diazo compound The sulfosalicylic acid is a moderately strong solid acid which provides the proper acidity for the reaction The sodium bicarbonate facilitates solution of the tablet on addition of water and the boric acid ..ts as a diluent and binding agent

2 The mat is composed of approximately one-half asbestos fibers and one-half cellulose fibers and is capable of adsorbing bilirubin When the mat is moistened with a few drops of urine containing bilirubin, the pigment remains on the outer surface of the mat By this means the bilirubin from a few drops of urine can be concentrated on a small surface area

3. The test is specific for bilirubin False positive reactions occur in only approximately 0 4 per cent of patients

Interpretation

1. A positive test is indicated by the rapid appearance of a blue or purple color on the mat around the tablet. The color which develops is stable over a considerable period and delayed color reactions are rarely observed so that *precise timing is not essential.* The intensity of the color reaction may be graded from ± (trace) to ++++. Color appearing on the tablet is disregarded. If no blue or purple color appears on the mat, the test is negative.

2. *The test is extremely sensitive and is capable of detecting* 0.125 μg. of bilirubin. By the use of a dilution procedure it is possible to determine roughly the amount of bilirubin present in urines containing high concentrations of the pigment. The urine is diluted with water until a faint positive test is obtained. This dilution contains approximately 0.05 mg. per 100 ml. and the concentration may be calculated by the formula

Dilution factor × *0.05* = Conc. *in mg /100 ml.*

3. Traces of protein give a pink color which is readily distinguished from the color produced by bilirubin However, the presence *of protein reduces the intensity of color at all concentrations of bilirubin* and reduces the sensitivity of the test.

4. When urine is exposed to room temperature, there is a progressive loss of detectable bilirubin. Urine must be analyzed within 30 minutes after voiding or stored in the refrigerator. At 5 C. no loss occurs over a period of 16 hours.

Reagents

The tablets and mats are marketed under the trade name of "Ictotest" and may be purchased from the Ames Company, Inc., 819 McNaughton Ave., Elkhardt, Ind

REFERENCES

1. Free, A. H and Free, H M A simple test for bilirubin Gastroenterology *24*: 414, 1953
2. Klatskin, G and Bungards, L An improved test for bilirubin in urine New England J Med *248*: 712, 1953
3. Sobotka, H, Luisada-Opper, A V., and Reiner, M A new test for bilirubin in urine Am J. Clin Path *23* 607, 1953

Hemosiderin

IRON SULFIDE TEST

Technic

1. Centrifuge a specimen of urine and place one drop of urine sediment on a glass slide.
2. Add one drop of 30 per cent aqueous ammonium sulfide solution.
3. Mix the two drops together and cover with a cover glass.

Comment

The iron in the hemosiderin reacts with ammonium sulfide to form black granules of ferric sulfide of varying sizes. The black granules may be found separately or included in epithelial cells, casts, or amorphous debris. Hemoglobin does not give a positive test

PRUSSIAN BLUE REACTION

Technic

1 Centrifuge 15 ml. of urine in a graduated 15 ml centrifuge tube.
2 Draw off and discard the supernatant solution down to the 1 ml mark.
3 Suspend the sediment in the supernatant solution.
4 Add an equal volume of 5 per cent hydrochloric acid and mix
5 Add 0 5 ml of a 10 per cent aqueous solution of potassium ferrocyanide and mix
6. Examine a drop under microscope

Comment

1 Hemosiderin appears as deep blue particles in the sediment If the color is not visible grossly, the sediment should be examined microscopically. Hemosiderin appears as blue granules within epithelial cells, as amorphous sediment, and occasionally as blue-pigmented casts.

2. The amount of hemosiderin may be graded as follows: 1+, blue-stained granules visible microscopically but not grossly; 2+, blue granules in the tip of the centrifuge tube; 3+, a blue button in the tip of the centrifuge tube; 4+, blue pigment visible before centrifugation.

Interpretation

Hemosiderinuria occurs (a) in patients with intravascular hemolysis, and (b) in patients with hemochromatosis. Except in patients with hemochromatosis, hemosiderinuria is usually associated with hemoglobinemia. Hemosiderinuria is associated with hemoglobinuria in patients with a marked degree of intravascular hemolysis.

Reagents

Ammonium sulfide solution (30 per cent)
Ammonium sulfide (conc.) 30 ml.
Distilled water 70 ml.

Hydrochloric acid (5 per cent)
Concentrated hydrochloric acid 52.5 ml.
Distilled water to make 400 ml.

Potassium ferrocyanide solution (10 per cent)
Potassium ferrocyanide solution 10 Gm.
Distilled water to make 100 ml.

REFERENCES

1. CROSBY, W. H. AND DAMESHEK, W. The significance of hemoglobinemia and associated hemoglobinuria with particular reference to various types of hemolytic anemia. J. Lab. & Clin. Med. *33*: 829, 1951.
2. HAM, T. H. Hemoglobinuria. Am. J. Med. *18*: 990, 1955.

Porphobilinogen

Technic

1. To 5 ml. of freshly voided urine in a test tube add 5 ml. of Ehrlich's reagent and mix.

2. Add 10 ml. of a saturated aqueous solution of sodium acetate and mix.

3. Add 3 to 5 ml. of chloroform and shake for several minutes.

4. Allow the tube to stand until the chloroform has separated out on the bottom.

Interpretation

1. Porphobilinogen, urobilinogen, and indole all form red-colored aldehyde compounds with p-dimethylaminobenzaldehyde (Ehrlich's reagent). The porphobilinogen aldehyde compound is insoluble in chloroform and remains in the aqueous (upper) layer. The urobilinogen and indole aldehyde compounds are soluble in chloroform and consequently are extracted into the lower chloroform layer. Thus, when the red color remains in the upper aqueous layer, the test is positive for porphobilinogen

2. In carrying out the test it is important to note that the reagents must be added in the order given above, the solution must contain the concentration of sodium acetate as achieved by the above proportions, and the chloroform extraction requires a very thorough shaking with the aqueous fraction. Red-colored globules of chloroform frequently adhere to the sides of the tube in the upper portion giving a false color to the aqueous layer. Reflections of the reddish color from the lower (chloroform) layer into the aqueous layer must be discounted. In the presence of large amounts of urobilinogen, a small fraction of the aldehyde compound may not be extracted from the aqueous layer by the chloroform. Under these circumstances a residual trace of color in the supernatant aqueous layer cannot safely be interpreted as porphobilinogen When the test is positive, the aqueous layer is an intense red color.

3. A positive test is strong presumptive evidence for the diagnosis of acute intermittent porphyria (table 58). The test is positive in approximately one-third of the patients with a "mixed type" of porphyria. The test is negative in erythropoietic (congenital) porphyria, chronic (cutanea tarda) porphyria, plumbism, and cirrhosis of the liver When the test is carried out properly, false-positive reactions are extremely rare.

TABLE 58.—*Clinical Manifestations and Laboratory Findings in Various Forms of Porphyria and Plumbism**

	Erythropoietic Porphyria	Hepatic Porphyria			Plumbism
		Acute Intermittent	*Cutanea* Tarda	Mixed Type	
Age	*Infants*	*Adults*	*Adults*	*Adults*	*All Ages*
Photosensitivity	Present	Absent	Present	Present	Absent
Red teeth and bones	Present	Absent	Absent	Absent	Absent
Psychiatric disturbances	Absent	Usually present	Absent	Absent	Present
Abdominal symptoms	Absent	Usually present	Absent	Occasionally	Present
Neurologic signs	*Absent*	*Usually present*	*Absent*	*Occasionally*	*Present*
Color of urine	Red	Brownish-red†	Red	Usually normal, rarely red	Normal
Porphobilinogen	*Negative*	*Positive*	*Negative*	*Positive in ⅓ of patients*	*Negative*
Uroporphyrin zinc-complex	Absent	Present	Present	Usually present	Absent
Coproporphyrinuria	Present	Present	Present	Present	Present

*Modified from S. Schwartz, Clinical Aspects of Porphyrin Metabolism, Veterans Adm Technical Bulletin, TB-10-94, Dec 1, 1953

†The color of the urine varies from brown to red and is often described as "port wine" Frequently the fresh urine is more or less normal in color, but on standing in the light slowly darkens so that in a few hours it is deep brownish red or even black This is due to porphyrin precursors and other chromogens of unknown constitution which are slowly changed in light and air to the corresponding porphyrins and pigments To produce the change rapidly the urine may be boiled for 30 minutes, the darkening starts in a few minutes and is complete in half an hour. A similar color change will occur in the urine of patients with alkaptonuria or melanuria

Reagents

See p. 223

REFERENCE

WATSON, C. J. AND SCHWARTZ, S A simple test for urinary porphobilinogen Proc Soc. Exper. Biol & Med. 47 393, 1941

Uroporphyrin Zinc-Complex

Technic

When viewed in a direct-vision spectroscope, the urine exhibits two well-defined absorption bands at approximately 580 and 542 mμ. Upon acidification of 10 ml. of urine with a few drops of concentrated hydrochloric acid there is an immediate shift of the bands to approximately 600 and 556 mμ.

Interpretation

1. When the uroporphyrin zinc-complex is present in low concentrations or when the urine is highly pigmented, the characteristic bands may not be visualized in a hand spectroscope.

2. Hemoglobin gives a somewhat similar absorption spectrum and its presence must be excluded by appropriate tests before the test can be considered as positive

3. Uroporphyrin is usually excreted as the zinc-complex in patients with acute intermittent and chronic mixed types of porphyria (table 58). In patients with erythropoietic (congenital) porphyria the uroporphyrin is excreted in the free state and the characteristic uroporphyrin zinc-complex spectrum is absent.

REFERENCES

1 SCHWARTZ, S · Clinical aspects of porphyrin metabolism Veterans Adm Tech Bull TB 10 94, Dec 1, 1953, Gov Printing Office, Washington 25, D C

2 WATSON, C J Porphyria In Adv Int Med, edited by W Dock and I Snapper, New York, Year Book Publishers, Inc, 1954, vol 7

Coproporphyrin

Technic

1. To 10 ml. of urine in a separatory funnel add 4 drops of glacial acetic acid
2. Add 2 volumes of ethyl ether (C.P.).
3. Shake for 3 minutes
4. Draw off and discard the lower aqueous phase.

5. Add 5 ml. of distilled water, shake for 30 seconds, draw off and discard the lower aqueous layer.
6. Repeat the water washing two more times.
7. Add 10 ml. of 5 per cent hydrochloric acid.
8. Shake for 3 minutes.
9. Draw off the lower acid layer into a test tube and examine for red fluorescence under a Wood's lamp* (Ultraviolet).

Interpretation

1. Normal urine when studied in the above fashion gives little or no fluorescence. If brilliant red fluorescence appears, especially when the hydrochloric acid is diluted 1:10 or 1:25, the presumptive test for coproporphyrinuria is positive. A control (normal) urine should always be run simultaneously and the highest dilution of the *unknown which will give a comparable degree of fluorescence should* be determined.

2. This test is markedly positive in plumbism and in patients with porphyria (table 58). Coproporphyrinuria, of lesser degree, is also present in patients with liver disease, pernicious anemia, aplastic anemia, infectious diseases, acute rheumatic fever and poliomyelitis.

Reagent

Hydrochloric acid, 5 per cent (1.5 N)
Concentrated hydrochloric acid (38 per cent) 52.5 ml.
Distilled water to make 400 ml

Hemoglobin, Methemoglobin and Methemalbumin

Pipet two ml of urine into each of four small test tubes (Wasserman tubes). If the urine contains sediment it should be centrifuged before use. Examine as outlined under "Methemalbumin" in the section on "Examination of Blood Pigments Related to Hemo-

*A very satisfactory lamp, the "Inspectolite" may be purchased from the Hanovia Chemical and Manufacturing Co., Newark, N. J.

globin" (p 212). The total heme pigments in urine may be quantitated by the method used for the determination of hemoglobin in serum (p. 213).

Examination of Stool Pigments Related to Hemoglobin

Urobilinogen

Technic

1. Place 10 Gm of a single well mixed specimen of stool and 90 ml of water in a Waring blender and mix for about three minutes.

2. While the specimen is being homogenized prepare a fresh 20 per cent solution of ferrous sulfate by adding 20 Gm. of hydrated ferrous sulfate ($FeSO_4 \cdot 7H_2O$) to 92 ml. of water in a 500 ml Erlenmeyer flask

3. Transfer the stool homogenate to the Erlenmeyer flask containing the freshly prepared ferrous sulfate solution.

4 Rinse out the Waring blender vessel with four successive 25 ml volumes of water and add these to the flask

5. Slowly and with gentle shaking, add 100 ml. of 10 per cent sodium hydroxide to the contents of the Erlenmeyer flask

6 Stopper the flask and place it in the dark for one hour or until the supernatant solution is nearly colorless.

7. Filter a small aliquot (about 10 ml) of the solution.

8. Pipet 1 25 ml of the filtrate into each of two 5 ml tubes accompanying the comparator block

9 To one tube add 1 25 ml of Ehrlich's reagent, mix the contents by inversion, and approximately 15 seconds later, add 2 5 ml of a saturated aqueous solution of sodium acetate and mix thoroughly

10 To the other tube, which is to serve as the blank, the reagents are added to the filtrate in reverse order to prevent color develop-

ment. First 2.5 ml. of the solution of sodium acetate is added and the contents are mixed thoroughly. With constant shaking, slowly add 1.25 ml. of Ehrlich's *reagent.*

11. The blank and reaction tubes are placed in two adjacent openings in the comparator block, and the sliding scale of dye standards is adjusted as necessary to bring the standard first selected for matching behind the blank and a vial of water behind the unknown tube. If the match of colors obtained by holding up the set in front of a light is not satisfactory, a stronger or weaker color standard is moved into position behind the blank until the best match is found. Interpolation may be made if it is evident that the color intensity lies between the two standards. If the blank develops a pink color or if the intensity of the unknown is higher than that of the most concentrated standard, the filtrate should be diluted 1:10 with water. Since matching the densities of color is most difficult in the lower range (0.1 mg. per 100 ml. and below), it is advisable to use the dilution of filtrate, if possible, which will produce densities comparable to those found in the higher range on the block.

12. The total amount of urobilinogen is calculated as follows·

$$\begin{matrix}\textit{Standard} \\ \text{reading} \\ (\text{mg./100 ml.})\end{matrix} \times 160 \times \begin{matrix}\textit{Any} \\ \text{Further} \\ \text{Dilution}\end{matrix} = \text{Ehrlich units per 100 Gm. of feces.}$$

Example: Standard reading, 0 5; filtrate diluted 1·10

0.5 × 160 × 10 = 800 Ehrlich units 100 Gm of feces

Precautions

1. These are the same as described under the determination of urobilinogen in urine (p 221).

2. It should be noted that the ferrous sulfate solution must be prepared fresh each time

Interpretation

Normal human adults excrete from 50 to 300 Ehrlich units per 100 Gm. of feces. Values above 400 units per 100 Gm. indicate an increased rate of blood destruction. A single determination on one day is insufficient to prove the presence of complete biliary

obstruction, such as is characteristic of cancer of the biliary tract. If less than 5 units per 100 Gm. are observed on each of four random samples during as many days, then complete obstruction is likely. Values over 10 units indicate that some bile is gaining access to the intestine. Values between 5 and 10 units and 300 and 400 units are borderline and require serial determinations or a four-day collection with determination of the excretion of urobilinogen per diem by the quantitative method. The latter method is undoubtedly superior insofar as an exact study of hemoglobin metabolism is concerned. However, for routine clinical work, the Ehrlich determination on a random sample will often give all of the information needed.

Representative values in various types of jaundice are given in table 57.

Reagents

See p. 223

REFERENCES

See p 224

Hemoglobin

Technic

1. By the use of a wooden applicator, a thin layer of stool is smeared over an area of about one cm^2 in the center of a piece of clean filter paper
2. Add two drops of guaiac solution
3. Add two drops of glacial acetic acid.
4. Add two drops of hydrogen peroxide (3 per cent)

Interpretation

The result is positive if a blue or dark green color appears. The results may be graded roughly as follows: immediate deep blue, 4+; moderately deep blue color appearing in about 30 seconds,

2 or 3+; slight but definite blue color developing in 1 to 3 minutes, 1 or 2+; slowly developing faint blue color, trace; no blue color, negative. The blue color which develops fades progressively with time to green-blue and finally blue-green. A faint green color occasionally appears if the stool contains iron but no blood.

Comments

1. The reagents must be added in the above order and in the approximate volumes given.

2. If the result is negative, it must be established that the reagents are active in detecting hemoglobin. This is done by performing the test on one drop of diluted blood (one drop of normal blood in 25 ml. of water) added to a piece of filter paper.

3. Certain batches of filter paper may give a trace reaction. This can be easily checked by performing the test without adding the stool.

4. Care must be taken to avoid contamination of the filter paper with blood from the bench or glassware.

5. It must be stressed that this simple, crude test will not do the thinking for the clinician. The test must be performed on a number of stool specimens before it can be concluded that there is no gastrointestinal bleeding. Consideration must be given to the possibility that gastrointestinal bleeding occurred in the past but not during the few days preceding the examination or that a nonbleeding gastrointestinal lesion is present Finally, for unexplained reasons, either false positive or false negative tests may be obtained in a small number of patients

Reagent

Guaiac solution

Saturated solution of guaiac crystals in 95 per cent ethyl alcohol.

REFERENCES

1 Hoerr, S O, Bliss, W R, and Kalfeman, J Clinical evaluation of various tests for occult blood in the feces J A M A *111* 1213, 1939

2 Peranio, A and Bruger, M The detection of occult blood in feces including observations on the ingestion of iron and whole blood J Lab & Clin Med *38* 433, 1951

Other Examinations

Cryoglobulins

Technic

1. Withdraw approximately 10 ml. of blood from the patient in the fasting state.
2. Immediately place the blood in a water bath or incubator at 37 C. and allow it to clot.
3. Immediately after centrifugation, place about 5 ml. of serum in the refrigerator at 5 C.
4. Examine the tube after 30 minutes and thereafter once daily for six days for the presence or absence of a precipitate composed of discrete white particles
5. When and if such a precipitate forms, the serum is removed from the refrigerator and warmed to 37 C. If cryoglobulins are present, the precipitate dissolves immediately.
6. The degree of cryoglobulinemia may be graded roughly as follows. grade 1, precipitate appearing in 2 to 6 days; grade 2, precipitate appearing in 24 hours; grade 3, precipitate appearing immediately on cooling.

Comments

1. A precipitate which forms at 5 C. cannot be considered as a cryoglobulin unless it redissolves on warming to 37 C.
2. Care must be taken to avoid mistaking a delayed fibrin clot for cryoglobulin precipitate. A fibrin clot forms as a discrete mass with the appearance of a gel which will not dissolve on warming to 37 C.
3. If a precipitate forms on the surface as a white ring and the serum below remains clear, this is a lipid and not a cryoglobulin precipitate
4. If cryoglobulins are present in large amounts they may begin to precipitate out at 32 C. In such a case care must be taken to maintain the blood at 37 C. prior to the time the serum is refrigerated
5. Cryoglobulinemia has been described in patients with multiple

myeloma, Raynaud's syndrome, purpura, chronic lymphocytic leukemia, and a variety of other disorders. The most prominent symptoms attributable to cryoglobulinemia are sensitivity to cold, Raynaud's syndrome, purpura or urticaria and a tendency to excessive bleeding from the mucous membranes. Retinal vein "thrombosis," arthritis, and progressive deafness have also been described. Search for cold precipitable proteins should be made in all cases of peripheral vascular disease, of multiple myeloma, symptomatic purpura, oozing or hemorrhages from the mucous membranes without obvious cause, and in all patients who present symptoms referable to exposure to cold.

6. Cryoglobulins may be semiquantitated by filling a hematocrit tube with serum. The hematocrit is then placed in the refrigerator for 3 days, after which it is centrifuged for 30 minutes at 3000 R.P.M. in a refrigerated centrifuge at 4 C. The per cent volume of the sedimented and packed cryoglobulin is then read as a "cryocrit."

REFERENCE

MACKAY, I R., ERIKSEN, N., MOTULSKY, A G., AND VOLWILER, W: Cryo and Macroglobulinemia. Am J Med. 20 564, 1956.

Euglobulin Test

Technic

One drop of serum is allowed to drop into a cylinder filled with distilled water to a depth of about 25 cm.

Interpretation

1. The test is positive when a massive precipitate appears immediately and settles rapidly to the bottom of the vessel without formation of any diffuse cloudiness behind the sedimenting floccules. The precipitate is readily soluble in physiologic saline Diffuse cloudiness in the water is observed with many different pathologic

and normal sera and is not considered to be a positive test.

2. This test is positive in most patients with macroglobulinemia with a gamma-2 electrophoretic mobility. It is negative in patients with macroglobulinemia with an abnormal component with beta mobility.

3. The macroglobulinemia syndrome of Waldenström occurs chiefly in males over the age of 50 years and is characterized by lassitude, dyspnea, bleeding from the mucous membranes, edema, anemia, hepatosplenomegaly, lymphadenopathy, lymphocytosis, and atypical "lymphocytic" cells in the bone marrow.

REFERENCES

1 Laurell, C. B., Laurell, H., and Waldenström, J. Glycoproteins in serum from patients with myeloma, macroglobulinemia and related conditions Am. J Med. 22: 24, 1957
2. Waldenström, J Abnormal proteins in myeloma. *In* Adv Int. Med. edited by W Dock and I Snapper, Chicago, Year Book Publishers, Inc., 1952, vol 5

Bence-Jones Proteins

Technic

1 Make the urine slightly but definitely acid by the addition of dilute acetic acid. If the urine is cloudy, or if a precipitate is present, it should be filtered.

2. Place about 10 ml. of urine in a large pyrex test tube along with a thermometer

3. Heat the urine *slowly* in a water bath. Note the temperatures at which precipitates appear and disappear

4. Finally bring the urine to the boiling point over a free flame, and keep it boiling for at least two minutes

5. If a precipitate persists after boiling, filter the urine *as hot as possible* and observe any precipitation which may take place in the filtrate as the solution cools.

6 When the urine filtrate is cold (37 C.), repeat the whole process to observe the phenomenon after the removal of albumin

Interpretation

1. In a urine initially containing no albumin, or one from which albumin has been removed, the presence of Bence-Jones protein will be indicated by increasing turbidity when heated at lower temperatures (approximately 45 C. to 60 C.), followed by complete solution of the precipitate at higher temperatures (80 C to 100 C.), and reappearance of the precipitate on cooling to 85 C. to 65 C.

2. The urine must be heated in a water bath, slowly raising the temperature to 100 C. during a period of 15 to 20 minutes. More rapid heating may result in a false-negative reaction.

3. The albumin must be filtered off while the urine is boiling. This can be done by boring two holes in a rubber stopper. The funnel stem is inserted into one hole. A glass tube with an angle is inserted into the second. The rubber stopper is then fitted tightly into a wide, short tube. The entire tube and about two-thirds of the funnel are immersed in boiling water contained in a beaker. Boiling urine is then poured onto the filter paper and albumin-free urine is collected in the collecting tube below.

4. If the above test is negative for Bence-Jones proteins and the diagnosis of multiple myeloma is suspected, it is desirable to add several drops of Pandy's reagent (saturated aqueous solution of phenol made by melting 6.7 Gm. of phenol and dissolving in 100 ml. of water) to the albumin-free urine and repeat the test This may enhance the detection of small amounts of the protein.

5. Bence-Jones protein will be found in the urine in approximately 40 to 47 per cent of the cases of multiple myeloma Bence-Jones protein has very rarely been found in disorders other than multiple myeloma: leukemia, polycythemia vera, carcinoma in bone, senile osteomalacia, multiple bone fractures and tuberculosis.

REFERENCES

1 Adams, W. S., Alling, E. L., and Lawrence, J S Multiple myeloma Am J Med 6 141, 1949

2. Sankuhler, S A simple and sensitive test for Bence Jones protein Am J Clin Path 22: 282, 1952

Heterophil Antibody Test for Infectious Mononucleosis

PRESUMPTIVE TEST

Technic

1. Serum is obtained from clotted blood (5 to 10 ml) Prior to use it must be inactivated for 30 minutes at 56 C.
2. Set up a row of 10 test tubes (10 x 75 mm.).
3. Add 0.4 ml. of physiologic saline to the first tube and 0.25 ml to the other tubes.
4. Add 0.1 ml. of inactivated serum to the first tube, and mix the contents of the tube.
5. Transfer 0.25 ml. of the contents of the first tube to the second tube, mix, etc until the last tube is reached. Mix and discard 0.25 ml. from the last tube.
6 Add 0.1 ml. of a 2 per cent suspension of washed sheep erythrocytes to each tube. Shake the tubes The final serum dilutions are 1 7, 1.14, 1 28, 1 56, 1:112, 1 224, 1:448, 1.896, 1:1792 and 1.3584.
7. Let the tubes stand at room temperature for 2 hours Shake the test tubes to resuspend the cells and read for agglutination with the naked eye If no agglutination is visible in any of the tubes, it is desirable to place the tubes in an almost horizontal position on the stage of a microscope and read with a low power objective (25 or 35 mm scanning lens)

DIFFERENTIAL TEST

Technic

1 Set up two test tubes (13 x 85 mm)
2. Add 1 ml of a thoroughly shaken suspension of guinea pig idney antigen to the first tube and 1 ml. of a thoroughly shaken uspension of beef cell antigen to the second tube.

3. Add 0.2 ml. of inactivated serum (56 C. for 30 minutes) to each tube.

4. Shake the tubes well and let stand for 3 minutes.

5. Centrifuge at 1500 R.P.M. for 10 minutes.

6. Remove the supernatant fluids with a capillary pipet. Make sure to transfer only clear supernatant fluid without particles.

7. Set up two rows of ten tubes each (10 x 75 mm.).

8. Add 0.25 ml. of physiologic saline to all tubes except the first tube in each row.

9. Add 0.25 ml. of the serum absorbed with guinea pig kidney antigen to each of the first two tubes in one row and mix.

10. Add 0.25 ml. of the serum absorbed with beef cell antigen to each of the first two tubes in the second row and mix.

11. Transfer 0.25 ml. of the contents of the second tube in each row to the third, mix, etc. Discard 0.25 ml. from the last tube.

12. Add 0.1 ml. of a 2 per cent suspension of sheep cells to each tube in both rows. Shake the tubes. The final serum dilutions are 1:7, 1:14, 1:28, 1:56, 1:112, 1:224, 1:418, 1:896, 1:1792, and 1:3584.

13. Let the test tubes stand at room temperature for 2 hours. Shake the tubes to resuspend the cells and read for agglutination with the naked eye. If no agglutination is visible in any of the tubes it is desirable to place the tubes in an almost horizontal position on the stage of a microscope and read with a low power objective.

Comments

1. A 2 per cent boiled suspension of horse kidney can be substituted for the guinea pig kidney antigen.

2. It is recommended that every time new reagents are used, a positive control serum of known titer be run The use of a positive control serum in the performance of the tests assures that the technic is being performed correctly and serves as a check on the potency of the reagents.

Interpretation

1. Heterophilic antibodies are antibodies that have the ability

to react with antigens that are apparently unrelated to those that stimulate their production. At least three distinct heterophil antibodies can be distinguished in human subjects, namely, (1) those which occur in normal human subjects in low titer (Forssman type), (2) those which occur in patients with serum sickness, and (3) those present in the serum of patients with infectious mononucleosis.

2. The unabsorbed heterophil titer (presumptive test) is rarely higher than 1:112 in normal subjects (table 59). In patients with such diseases as virus hepatitis, virus pneumonia, lymphoma, leukemia, polycythemia, or tuberculosis the titer may be as high as 1:896. The titer may be as high as 1:1792 in patients with serum sickness or in patients with a recent history of serum sickness. In patients with infectious mononucleosis the titer is usually greater than 1:224. However, about 25 per cent of patients with this disease will have a titer (unabsorbed) between 1:7 and 1:224. A titer above 1:896 is almost invariably diagnostic of infectious mononucleosis. The titer is usually not significantly elevated in the first 5 days of the illness; maximum titers are obtained during the seventh to twenty-first day of the illness; and the titer usually returns to normal 4 to 8 weeks after the onset of the illness. The titers may be elevated for as long as 10 months.

3. The indications for the differential heterophil test are as follows: (a) in patients suspected of having infectious mononucleosis but with a titer, as determined by the presumptive test, of 1:112 or less, (b) a titer of agglutinins of 1:56 or higher in patients without clinical or hematologic findings of infectious mononucleosis; and (c) a history of a recent injection of horse serum in a patient with a titer of 1:56 or higher, as determined by the presumptive test.

4. The differential heterophil test is based on the observations that the Forssman type antibody and the antibody observed in patients with serum sickness are absorbed by both guinea pig kidney and beef erythrocytes. The heterophil antibody of infectious mononucleosis is absorbed by beef erythrocytes but not at all, or incompletely, absorbed by guinea pig kidney. Thus, infectious mononucleosis is the only disease in which antisheep agglutinins have the following behavior: at least one-eighth of the original titer of antisheep agglutinins remains after absorption with guinea

pig kidney (i e., in twofold serial dilutions the drop does not exceed 3 tubes) *and the antisheep agglutinins are completely removed after* absorption with beef red cells. Interpretation of the differential test is summarized in table 59. Examples are given in table 60.

Reagents

Guinea pig kidney antigen, beef erythrocyte antigen, sheep erythrocytes, and positive control serum may be purchased from a number of sources and are entirely satisfactory. Examples of satisfactory sources are as follows: American Hospital Supply Corp., Evanston, Ill. (these products are prepared by the Mount Sinai Medical Research Foundation, 2750 West 15th Place, Chicago, Ill., under the direction of Dr. I. Davidsohn), Ortho Pharmaceutical Corporation, Raritan, N. J., and Certified Blood Donor Service, Inc., 146-16 Hillside Avenue, Jamaica 35, N. Y.

TABLE 59—*Differential Test for Infectious Mononucleosis*

Group		Heterophil Antibody Titer	Heterophil Antibody Absorbed by	
			G P Kidney	Beef Erythrocytes
Normal	90%	1:7 — 1:28	Complete	Complete or Partial[3]
	10%	1:56 — 1:112		
Other Diseases[1]		1:7 — 1:896	Complete	Complete or Partial[3]
Serum Sickness		1:7 — 1:1792	Complete	Complete
Infectious Mononucleosis	25%	1:7 — 1:224	Incomplete[2]	Complete
	75%	1:224 — 1:2 400,000		

[1]Virus hepatitis, virus pneumonia, lymphoma, leukemia, polycythemia, tuberculosis, etc.

[2]Incomplete removal in about ⅓ of individuals

[3]The titer may decrease 1 to 4 tubes (2 fold dilutions)

TABLE 60—*Examples of Titers in the Differential Agglutination Test When the Presumptive Test is Not Diagnostic*

Titer of			Result
Presumptive Test	Differential Test After Absorption With		
	Guinea Pig Kidney	Beef Red Cells	
1 224	1 112	0	Positive for I M
1 224	1 28	0	Positive for I M
1 224	0	0	Negative for I M
1 224	0	1 112	Negative for I M
1 56	1 56	0	Positive for I M
1 56	1 7	0	Positive for I M
1 56	0	0	Negative for I M
1 56	0	1 7	Negative for I M
1 28	1 28	0	Positive for I M
1 28	1 7	0	Positive for I M
1 28	0	0	Negative for I M
1 28	0	1 7	Negative for I M

REFERENCES

1 DAVIDSOHN, I Test for infectious mononucleosis Am J Clin Path 8 56, 1938
2 ——, STERN, K, AND KASHIWAGI, C The differential test for infectious mononucleosis Am J Clin Path 21 1101, 1951
3 —— AND GOLDIN, M The use of horse kidney in the differential test for infectious mononucleosis J Lab & Clin Med 45 561 1955

Reagents, Miscellaneous

Standard Solutions of Hydrochloric Acid

Accurately standardized, stable solutions of hydrochloric acid are prepared commercially and can be purchased through many

sources (for example, Fisher Scientific Company, St. Louis, Mo.). It is not recommended that the physician or student prepare his own standard solutions.

Physiologic Saline (0.85 per cent sodium chloride)

Sodium chloride 8.5 Gm.

Distilled water to make 1000 ml.

The sodium chloride must be chemically pure (C.P.) and must be dried in a desiccator prior to weighing. If the solutions are stored in glass-stoppered, tightly closed bottles they will remain standard for months.

Cleaning Solution

Dissolve 100 Gm. of powdered potassium dichromate in about 200 ml. of water. With caution, slowly add about 800 ml. of technical grade sulfuric acid to the dichromate solution in a pyrex container.

Silicone Glassware

Needles are prepared by routine cleaning methods and then boiled in a 1 per cent solution of "Arquad—2C" (Armour) for 2 minutes. They are then rinsed with distilled water, allowed to dry and may be autoclaved as desired. The silicone surface should be applied after each use. Arquad is removed by washing the needle in soap and warm water.

Syringes are prepared by placing a small amount of Dow-Corning* Silicone Stopcock Grease in the bore of the syringe. The grease is spread by moving the bore in the syringe.

Centrifuge tubes, test tubes and other glassware are prepared by dipping them in a 1:100 dilution of Dow-Corning Z 4141 silicone solution, followed by a water rinse. The water rinse removes excess solution from the glass surface, and thereby prevents spotting. The glassware may then be dried in the air for 24 hours or dried at 100 C. for 10 minutes. Glassware prepared in this manner may be washed a number of times before it must be coated again

*Midland, Michigan

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